MONOGRAPH ON CHOLINE SALTS #61

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M O N O G R A P H

O N

C H O L I N E S A L T S

TR-72-1552-24

Submitted Under:
Contract No. FDA 72-104

May 18, 1973

INFORMATICS INC.
6000 Executive Boulevard
Rockville, Maryland 20852

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CHOLINE SALTS

Summary

The biochemical functions and physiological aspects of the choline salts, choline chloride and choline bitartrate, have been well established since the 1950's. However, most feeding studies have been directed towards deficiency and determining the optimal dietary levels of choline. Little work has been performed to investigate possible deleterious effects from long term feeding of higher choline levels for various species.

Reidesel and Hines (80) reported that choline was absorbed unchanged from the small intestine of rats, possibly through enzymatic absorption. Choline is stored in the animal in the form of the phosphatides, lecithin and sphingomyelin. Since the concentration of choline in animal tissues is proportionate to their phospholipid content, the best sources are nerve tissue, liver, kidneys, brain, heart, and egg yolk (34).

The three major metabolic functions of choline involve transmethylation, transacetylation, and the production of phospholipids. Transmethylation involves the transfer of one of the labile methyl group attached to the nitrogen to an acceptor molecule. To synthesize methionine, one methyl group from choline is transferred to homocysteine to yield methione and ethanolamine. One of these methyl groups may also be transferred to guanidoacetic acid to produce creatine for the organism (34).

Transacetylation occurs when an acetyl group from acetyl-coenzyme A is transferred to choline to give acetylcholine, a nuerohormone (4). The production of the phospholipid, lecithin, takes place in the mitochondria (59). Choline and ATP react to form phosphorylcholine, which is then reacted with cytidine triphosphate to yield cytidine diphosphate-choline. The choline is transferred to 1,2-diacylglycerol to produce phosphatidyl choline, commonly known as lecithin.

De la Huerga and Popper (21) reported that 0.3% of an oral dose of choline to man was excreted as choline in the urine. Sixty to sixty-seven precent of the choline nitrogen was excreted in the urine as trimethylamines, resulting from microbial breakdown of choline in the large intestine.

Dyer and Wood (24) reported that all strains of Proteus rettgeri, P. ichthyosmius, P. vulgaris, P. mirabilis, and Shigella alkalescens produced trimethylamine from choline.

Choline has been found to be essential for normal growth in the rat, chick, and dog; symptoms of choline deficiency are well known (34). Since choline lacks the specificity characteristic of vitamins and is a structural component of various tissue, Hawk et al. do not consider it to be a vitamin.

Several authors (81, 34, 97, 79, 13, 53, 100) have reported a lipotropic effect from dietary choline in quail, chicks, mice and rats. In the absence of dietary choline, nuetral fat and cholesterol esters accumulate in the liver. Choline chloride also reduced plasma cholesterol levels in rats (16, 94). Yamaguchi reported the reduction of the incidence of experimental arteriosclerosis in chicks fed on a high cholesterol diet and treated orally with choline chloride (100). Rabbits fed a high cholesterol diet suffered high plasma cholesterol levels and atherosclerosis; dietary choline chloride prevented these symptoms (28).

The feeding studies have been arranged by species for comparison. Again, no long term studies are available and very few short term studies have been performed. A table of acute toxicity has been prepared on page 9.

Symptoms of acute toxicity in mice and rats are characterized by salivation, trembling, jerking, convulsions, and respiratory paralysis (39, 37, 74). Bleeding from the eyes was also reported.

Fritz et al. (29) reported 0.15% choline chloride to optimize growth in chicks, but an 0.19% level protected completely against perosis. Marvel et al. (63) found 0.15 choline chloride to maximize growth in chicks, also.

After a complete histological examination of rats fed 0.01, 1, 2.7, 5, and 10% choline chloride for 3-4 months, Hodge (38) concluded that no consistent histopathological changes were attributable to choline chloride. Hodge (38) gave rats 0.01, 1.0, 2.7, 4, 5, 6.7, and 10% choline chloride as drinking water for 2 months; water consumption decreased at the 2.7% level. Mortality increased from the 5% to the 10% levels. Roth and Allison (82) found 1.35% choline chloride to be tolerated by rats in a 20 day study. Higgins et al. (36) reported a bronze-coloring effect to rats upon oral administration of choline chloride.

Rabbits required 0.12% choline chloride to prevent deficiency symptoms (42).

Doses of 10 mg/kg choline chloride daily (later 2 and 3 times daily) produced macrocytic and hyperchromic anemia in 40 dogs (20).

The daily intake of man on an average diet has been found to range from 150-600 mg choline per day (87).

CHOLINE BITARTRATE

Chemical Information

I. Nomenclature

A. Common Name

Choline Bitartrate

B. Chemical Names

(2-Hydroxyethyl)trimethylammonium bitartrate

C. Trade Name

None

D. Chemical Abstracts Registry Number

000087672

II. Empirical Formula

$C_9H_{19}NO_7$

III. Structural Formula

$(HOCH_2CH_2N^+(CH_3)_3)\ HC_4H_4O_6$

IV. Molecular Weight

253.25

V. Specifications

Food Chemicals Codex	
Assay	Not less than 98.0% of $C_9H_{19}NO_7$, calculated on the anhydrous basis
Limits of Impurities	
Arsenic (as As)	Not more than 3 ppm (0.0003%)
Heavy metals (as Pb)	Not more than 20 ppm (0.002%)
Lead	Not more than 10 ppm (0.001%)
Residue on ignition	Not more than 0.1%
Water	Not more than 0.5%

VI. Description

A. General Characteristics

Choline bitartrate is a white, hygroscopic, crystalline powder

having an acidic taste. It is odorless or may have a faint trimethylamine-like odor.

B. Physical Properties

Choline bitartrate is freely soluble in water, slightly soluble in alcohol, and insoluble in ether, chloroform, and benzene.

C. Stability

Store in tight containers.

VII. Analytical Methods

Choline is used in many commercial products. The official method for the determination of choline in drugs is a microchemical test. The sample is dissolved in an ammoniacal silver nitrate solution and the choline is extracted with a suitable immiscible solvent. After evaporation, a little of the residue is dissolved in hydrochloric acid; this is then diluted with water to make a 1:1000 solution. A drop is placed on a glass slide. One drop of acetone is stirred in. Then one drop of reinecke salt is stirred in. Under a magnification of 100-150X, thin, hexagonal plates and star shaped forms are seen. If the solution has been diluted to 1:1000 - 1:10,000, six sided; coffin-shaped plates with occasional rosette aggregates of plates on edge are seen. Another test involves placing a drop of the 1:100 sample on the slide and adding 1 drop of platinic chloride, stirring. A small drop of sodium iodide is then added without stirring. Small black rectangular prisms and slender black rods are visible (2).

VIII. Occurrence and levels found in:

A. Plants

Choline is present in many plants, e.g., hops, belladonna, and strophanthus.

B. Animals

Choline is found in many animal organs, e.g., bile, brain, and egg yolk. It is the main constituent of lecithin.

C. Synthetics

Choline is usually made synthetically from trimethylamine and ethylene chlorohydrin or ethylene oxide.

The following tables indicate choline content of different dietary sources (87).

TABLE VII

TOTAL CHOLINE CONTENT OF ANIMAL PRODUCTS[1]

Product	Choline chloride, mg./g. Fresh	Dry
Pig		
Adrenals	5.88	
Liver	5.52	18.35
Spinal cord	4.27	13.67
Brain	3.75	18.20
Pancreas	3.29	12.60
Kidney, No. 1	3.17	14.10
Kidney, No. 2	2.56	13.06
Ovary	2.78	17.38
Heart	2.31	11.16
Spleen	2.08	10.01
Small intestine	1.65	14.86
Tongue, No. 1	1.39	5.41
Tongue, No. 2	1.36	4.86
Shoulder, No. 1	1.05	2.29
Shoulder, No. 2	0.86	2.03
Ham	0.88	2.00
Chops	0.77	1.81
Lard	—	<0.05
Chicken		
Egg yolk	17.13	32.81
Liver	3.42	12.50
Heart	2.36	10.40
Kidney	2.23	11.32
Egg albumen	—	<0.05
Lamb		
Kidney	3.60	17.82
Shoulder	1.19	3.07
Chops	1.07	3.27
Beef		
Veal liver	6.52	22.72
Beef liver	6.30	20.47
Veal kidney	3.48	15.00
Beef kidney	3.33	16.32
Veal rib roast	1.13	3.44
Beef roundsteak	0.95	3.53
Beef rib roast	0.82	2.44
Milk		
Skim milk powder	1.59	1.63
Whole milk powder	1.07	1.10
Cheddar cheese	0.48	0.70
Fresh milk	0.147	1.14
Commercial casein	—	<0.05
Butter	—	<0.05
Fish		
Fish meal	3.29	3.47
Trout muscle	0.87	4.89
Red snapper muscle	0.84	4.12
Cod liver oil	—	<0.05
Miscellaneous		
Liver extract	15.93	16.36
Extracted liver residue	4.39	4.50
Liver sausage	2.67	5.52
Tankage	2.31	2.65
Meat meal, No. 1	1.62	1.73
Meat meal, No. 2	1.30	1.42
Bologna sausage	0.71	2.38

TABLE VIII

TOTAL CHOLINE CONTENT OF PLANT PRODUCTS[1]

Product	Choline chloride, mg./g. Fresh	Dry
Cereal grains		
Defatted wheat germ	4.23	4.53
Raw wheat germ, No. 1	4.10	4.40
Raw wheat germ, No. 2	4.03	4.32
Raw corn germ stock	1.60	1.78
Rolled oats	1.51	1.63
Wheat shorts	1.48	1.63
Wheat bran	1.43	1.56
Barley	1.39	1.55
Rice polish	1.26	1.36
Oats	0.94	1.00
Wheat	0.92	1.01
Polished rice	0.88	1.02
Molasses (blackstrap)	0.86	—
Wheatena	0.62	0.68
White flour	0.52	0.57
Corn meal (unbolted)	0.42	0.47
Yellow corn	0.37	0.41
Corn meal (bolted)	0.10	0.11
Non-leafy vegetables (sun-dried)		
Snapbeans	3.40	3.81
Green soybeans	3.00	3.32
English peas	2.63	2.90
Cowpeas	2.57	2.84
Asparagus	1.28	1.47
Vegetable oils		
Hydrogenated cocoanut oil	—	<0.05
Oleomargarine	—	<0.05
Refined corn oil	—	<0.05
Refined soybean oil	—	<0.05
Other seeds		
Cottonseed meal No. 1 (7.0% fat)	3.50	3.76
Cottonseed meal No. 2 (7.5% fat)	3.25	3.51
Soybean meal (2.5% fat)	3.45	3.75
Mature soybeans (19.5% fat)	3.40	3.58
Cottonseed kernels (36% fat)	2.98	3.19
Edible peanut meal	2.35	2.52
Peanut meal (6% fat)	2.26	2.44
Peanuts (Spanish) (43% fat)	1.67	1.74
Peanuts (Runner) (45.5% fat)	1.57	1.65
Peanut butter	1.45	1.48
Pecans	0.50	0.53
Root crops (sun-dried)		
Irish potatoes	1.06	1.31
Carrots	0.95	1.12
Turnips	0.94	1.11
Sweet potatoes	0.35	0.36
Leafy material (sun-dried)		
Mustard tops	2.52	2.77
Young cabbage	2.51	2.90
Turnip tops	2.45	2.69
Spinach	2.38	2.75
Rape	2.30	2.86
Pokeweed	2.28	2.64
Alfalfa leaf meal, No. 1	1.43	1.55
Alfalfa leaf meal, No. 2	1.22	1.31

TABLE IX

TOTAL CHOLINE CONTENT OF MEATS[2]

Sample	Choline, mg./100 g.			
	Fresh		Dry	
	Range	Avg.	Range	Avg.
Veal				
Leg	95–108	102	366–432	389
Roast leg	125–141	132	338–392	360
Shoulder	83–100	93	268–373	337
Roast shoulder	133–143	139	310–376	343
Sirloin chop	87–105	96	242–404	317
Braised chop	128–157	140	242–342	285
Shoulder chop	92–101	97	307–422	376
Braised chop	149–156	154	317–400	366
Stew meat	94–100	96	336–400	367
Cooked stew	137–149	142	360–378	370
Lamb				
Leg	75–92	84	262–317	290
Roast	122–124	123	284–295	290
Sirloin chop	75–77	76	179–198	189
Broiled chop	100–126	113	204–252	228
Stew meat	76–82	79	222–230	226
Cooked stew	116–128	122	247–291	269
Pork				
Ham	101–129	120		
Cured ham	98–129	122		
Beef				
Liver	470–570	510		
Round	65–70	68		
Tongue	108	108		
Heart	170	170		
Braised heart	200–275	238		
Kidney	240–284	262		
Brain	399–420	410		
Miscellaneous				
Bologna	60	60		
Frankfurters	57	57		
Pork links	48	48		
Canadian bacon	80	80		

TABLE X

TOTAL CHOLINE CONTENT OF GRAINS AND OF GRAIN PRODUCTS

(expressed as milligrams of choline chloride per 100 g. on a moisture-free basis)[3]

Variety		Milled fractions	
Hard spring wheat	91	Whole wheat	102
Hard winter wheat	79	Germ	354
Soft winter wheat	88	Bran	153
Soybeans	237	Low-grade flour	69
Oats	114	Bleached low-grade flour	69
Barley	110	Clear flour	61
Flax	107	Bleached clear flour	62
		Patent flour	70
		Bleached patent flour	69

CHOLINE CHLORIDE

Chemical Information

I. Nomenclature

A. Common Name

Choline chloride

B. Chemical Names

(2-Hydroxyethyl)trimethylammonium chloride

C. Trade Names

1. Biocolina
2. Hepacholine
3. Lipotril

D. Chemical Abstracts Registry Number

000067481

II. Empirical Formula

$C_5H_{14}ClNO$

III. Structural Formula

$(HOCH_2CH_2N+(CH_3)_3)Cl-$

IV. Molecular Weight

139.63

V. Specifications

Food Chemicals Codex	
Assay	Not less than 98.0% of $C_5H_{14}ClNO$, calculated on the anhydrous basis
Limits of Impurities	
Arsenic (as As)	Not more than 3 ppm (0.0003%)
Heavy metals (as Pb)	Not more than 20 ppm (0.002%)
Lead	Not more than 10 ppm (0.001%)
Residue on ignition	Not more than 0.05%
Water	Not more than 0.5%

VI. Description

A. General Characteristics

Choline chloride appears as colorless or white crystals or crystalline powder, usually having a slight odor of trimethylamine.

B. Physical Properties

Choline chloride is hygroscopic, and very soluble in water and in alcohol.

C. Stability

Must be stored in tight containers.

VII. Analytical Methods

See Choline Bitartrate.

VIII. Occurrence and Levels Found in:

See Choline Bitartrate.

CHOLINE SALTS

Biological Data

I. Acute Toxicity

Substance	Animal	No.	Route	Dosage in Mg/Kg Body Wt.	Measurement	Ref.
Choline Chloride	Mice	106	i.p.	320	LD_{50}	39
Choline Chloride	Mice	Unk.	i.p.	31.3	LD_{50}	65
Choline Chloride	Rats	151	i.p.	290-340 (a)	LD_{50}	37
Choline Chloride	Rats	118	i.p.	370-380 (b)	LD_{50}	37
Choline Chloride	Rats	131	i.p.	410-490 (c)	LD_{50}	37
Choline Chloride	Rats	114	i.p.	590-750 (d)	LD_{50}	37
Choline Chloride	Rats	90	p.o.	6700	LD_{50}	39
Choline Chloride	Rats	10-60	p.o.	3400 (e)	LD_{50}	74
Choline Chloride	Rats	10-60	p.o.	6100 (f)	LD_{50}	74
Choline Chloride	Rats	Unk.	p.o.	5100	LD_{50}	87
Choline Chloride	Rabbits	Unk.	s.c.	500	LD_{50}	65

(a) 200 mg/ml choline hydrochloride
(b) 100 mg/ml choline hydrochloride
(c) 40 mg/ml choline hydrochloride
(d) 20 mg/ml choline hydrochloride
(e) Data from 500 and 670 mg/ml choline chloride averaged
(f) Data from 200 and 400 mg/ml choline chloride averaged

A. Choline Chloride

Mice

Hodge and Goldstein reported an LD_{50} of 320 mg/kg for choline chloride injected i.p. into mice. Data is tabulated below:

No. of Mice	Dose mg/kg	No. of Dead	Percent of Mortality
16	250	0	0
15	280	4	27
15	300	7	47
15	330	6	40
15	350	10	67
15	400	13	87
15	500	13	87

Salivation, trembling, jerking, cyanosis, clonic shivering convulsions, and respiratory paralysis preceeded death. The fur around the eyes seemed wet; the eyes appeared to darken (39).

Hodge cited a lethal dose s.c. of 700 mg/kg of choline chloride in mice from Arai (39).

Rats

Hodge and Goldstein reported an LD_{50} of 6700 mg/kg choline chloride when administered by gavage to rats (150 g). Data is tabulated below:

No. of Rats	Dose mg/kg	No. of Dead	Percent of Mortality
15	2666	0	0
15	3600	3	20
15	4000	3	20
15	4466	6	40
15	5333	9	60
15	6660	12	80

Salivation, trembling, jerking, cyanosis, clonic shivering, convulsions, respiratory paralysis were noted. Bleeding from the eyes was observed in 60% of the dying rats. On autopsy, the blood vessels of the diaphragm and stomach were engorged. Stomachs were bleached and distended; the hearts ceased in diastole (39).

Hodge reported LD_{50}'s for the intraperitoneal injection of choline chloride into rats of the same weight. They are listed in the following table:

TABLE I.
Dosage-Mortality Table for Rats Grouped by Dosage Ranges.*
(Intraperitoneal Injection: 518 Rats.)

	Concentration of choline hydrochloride solutions							
	200 mg/ml		100 mg/ml		40 mg/ml		20 mg/ml	
Dosage range mg/100 g body wt	No. rats	% mortality	No. rats	% mortality	No. rats	% mortality	No. rats	% mortality
20-24	15	0						
25-29	44	25						
30-34	31	61	22	32				
35-39	46	72	43	54	8	12		
40-44	15	100	28	79	43	19		
45-49					39	41		
50-54			20	80	11	82	9	67
55-59			5	60			30	43
60-64					15	87	22	64
65-69							6	100
70-74							14	36
75-79							23	71
80-84					15	100		
85-89							14	86
Range of LD_{50}	29-34 mg/100 g body wt		37-38 mg/100 g body wt		41-49 mg/100 g body wt		59-75 mg/100 g body wt	

Changes in respiration in these rats were closely followed by trembling, convulsive movements, salivation, cyanosis, respiratory paralysis and death. Hemorrhage around the eyes occurred in 2/3 of the rats dying from the drug. Death ensued within 5 minutes, if the rat died at all (37).

Groups of 5 to 30 rats (male and female weighing from 76-343 g) were fasted for 24 hours and then given choline chloride by stomach tube. Four different concentrations were used (200, 400, 500, 670 mg/ml). The data from the two higher concentrations were combined to calculate an LD_{50} of 3400 mg/kg. The two lower concentrations produced an LD_{50} of 6100 mg/kg. Five to ten minutes after intubation, there was an initial excitement period, characterized by jerking movements and occasional convulsions. Bloody tears (chromodacryorrhea) appeared in 30-90% of the animals which succumbed. This was followed by a depression with complete relaxation and depressed respiration, which finally terminated in respiratory paralysis after 15 to 30 minutes. Animals which lived longer than 30 minutes usually survived (74).

An LD_{50} of 5100 mg/kg was reported for the oral administration of choline chloride to rats (150 g). Data was cited from McArthur and Lucas (87).

Rabbits

Citing Dreyfus (1920), Hodge and Goldstein reported a lethal dose of 1000 mg/kg choline chloride administered rectally to rabbits, 1.1 mg/kg intravenously, and 1000 mg/kg subcutaneously (39).

Cats

Citing from Lohman (1907) and Arai (1922), Hodge and Goldstein reported a lethal dose of 35 mg/kg choline hydrochloride injected i.v. into cats (39).

B. Choline Bitartrate

No Information Available

II. Short Term Studies

A. Choline Bitartrate

No Information Available

B. Choline Chloride

Chicks

Fritz et al. designed a series of experiments to establish the optimal choline chloride level for chicks on a cerelose-based diet containing 3480 calories metabolizable energy. Growth was maximized at 0.15% choline chloride. A level of 0.19% was necessary for complete protection against perosis. Higher levels of choline chloride were not tested (29).

Rapid growth in 25 chicks was reported by Marvel et al. when 0.15% choline chloride was added to a basal ration containing corn, soybean oil meal, alfalfa leaf meal, distillers' dried solubles, minerals and vitamins. Total choline content equalled 0.29% in the diet. The animals were weighed at 4 weeks. Growth was depressed without choline chloride supplementation; growth increased proportionately as the increments of choline chloride increased from 0 to 0.15% (63).

Mice

Ten young male DD mice (10 g) were fed a basal diet plus 400 mg % (8-12 mg/day) choline hydrochloride for 30 days; ten control animals were also studied. Feed was given at 2 g/mouse/day up to 10 g body weight, beyond which the amount was increased to 3 g; water was mixed in the feed. Growth differences were not significant. Some deficiency symptoms were noted in the control group, although the diet was supposedly sufficient in the B vitamins (59).

Rabbits

Hodge fed groups of 5 rats each, choline chloride at levels of 0.01, 1, 2.7, 5, and 10% for 3-4 months. An unspecified number of control rats were used as well. Growth was reduced by 20% at the 2.7% level, by 45% at the 5% level, and 100% at the 10% level. Food consumption was reduced at the 5 and 10% levels, definitly influencing growth rate. The brain, stomach, kidney, heart, lung, spleen, liver, small intestine, large intestine, and ovary or testis were examined histologically. Rats on the higher dosages tended to have smaller organs than the controls; however, animals on the 10% level had much larger organs than controls. High choline intake rats produced almost no fat in the subcutaneous tissues, the mesentery, or perirenal sites. The 1% group had an increased hemosiderin containing phagocytes. The liver of one 2.7% rat had an area of focal degeneration. All tissues of higher level rats were normal. It was concluded that no consistent histopathological changes could be attributed to choline chloride (38).

Choline chloride solutions containing 0.01, 1.0, 2.7, 4, 5, 6.7 and 10% by weight were substituted for the drinking water for groups of 5 rats. Growth was decreased at the 1% level and by 40% at the 2.7% level. No growth occurred at 4%, and on a 5% solution the survivors after 2 months weighed only 55 and 59% of their original weight. No rats consuming 6.7 or 10% solutions survived 8 weeks; 7 out of 10 died within the first 10 days. Water consumption was markedly decreased at the 2.7% level and higher. Sections of the brain, stomach, kidney, heart, lung, spleen, liver, small intestine, large intestine, and ovary or testis were examined histologically. Increasing percentages of choline produced small livers. In general, the findings were negative. An increased number of hemosiderin-containing phagocytes were found in the spleens of the 0.01% group. In the same group 3 out of 5 kidney sections revealed a few focal collections of lymphocytes in the cortex and pyramids (38).

Roth and Allison reported that 10 Sherman rats (250 g) tolerated a level of 1.35% choline chloride added to a basal diet containing 12% casein for 20 days. Food intake was restricted; hence, weight loss occurred. No hypertrophy of the kidney was noted (82).

In a series of experiments on rats, Higgins et al. found dietary choline to produce a bronzing effect on the rat's hair. In the first experiment, 150 mg (total) was administered both by stomach tube and in drinking water to young rats. A reddish-brown pigment, apparently adherent to the hair, first appeared around the base of the tail within a few days after administration; gradually the coloring extended forward to involve the entire animal in 2-3 weeks. Supplementation with yeast returned the rats to normal in 2 weeks. A protein diet enriched with either yeast or liver extract prevented the pigmentation as did dextrin (carbohydrate source). Growth was normal at all times (36).

Rabbits

Hove et al. reported that an 0.12% choline chloride added to a peanut meal-casein diet (choline deficient) prevented the onset of deficiency symptoms in rabbits (42).

Dogs

Davis administered 10 mg/kg choline chloride by stomach tube to 4 dogs for at least 25 days. After a definite anemia was detected, 10 mg/kg choline chloride was given twice daily. When a lower red cell level had been reached (unspecified time), 10 mg/kg choline chloride was given three times a day. One other dog was "accelerated" by being given 2 daily doses 7 days after initiation of the test, and 3 daily doses on the 26th day of the test. Two other dogs received 3 doses daily from the beginning. Erythrocyte numbers were reduced finally to 30%. Davis classified the anemia as being macrocytic and hyperchromic. He suggested that choline depresses erythropoiesis by increasing the blood and oxygen supply to bone marrow (20).

Man

Sebrell and Harris, citing Johnson et al. (1945), reported a daily intake of choline of 624-899 mg for a 5-day period (150 mg/day) for young American men. A figure of 250-600 mg/day was arrived at in Toronto. From Sweden came an estimate of 300-500 mg/day (87).

III. Long Term Studies

None Available

IV. Special Studies

None Available

I. Breakdown

No Information Available

II. Absorption - Distribution

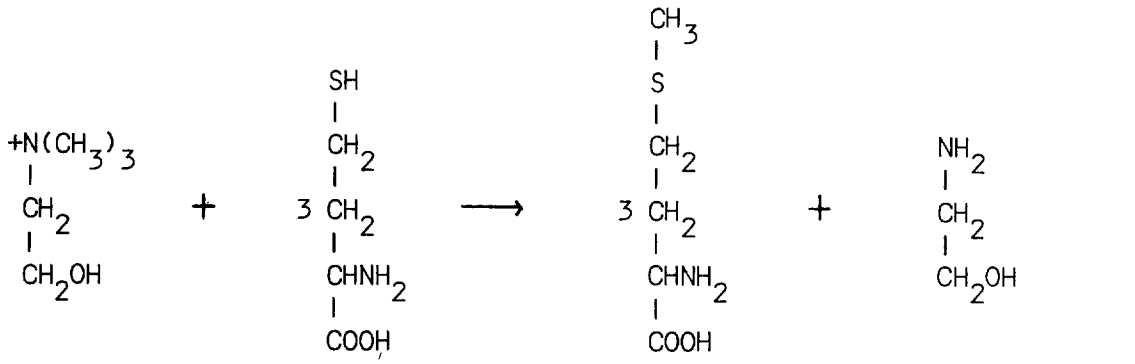
Riedesel and Hines reported that choline chloride was absorbed at approximately 400 mg/kg/hr when 200 mg/kg choline chloride was injected into the intestine of rats. Twenty-eight percent was absorbed within the first hour, with 39, 63, and 82% being absorbed by the second, third, and fourth hours. Since choline disappeared at a fairly constant rate, the authors suggested some type of enzymatic absorption. When choline was incubated with rat intestinal homogenate (for the same length of time as required for 39% absorption in vivo), the choline concentration did not decrease. When intestinal bacteria were suppressed by dietary antibiotics, choline disappeared at the same rate. The authors concluded that choline was absorbed unchanged from the small intestine, and that intestinal bacteria must play a minor role, if at all, in its disappearance (80).

Choline is stored in the animal in the form of phosphatides (lecithin and sphingomyelin). Phosphatides are the major components of the fatty matter of the brain, kidney, and liver. Sphingomyelins contain a fatty acid, sphingosine, and a choline-phosphoric acid group. Lecithin (phosphatidyl choline) is the glyceryl ester of a pair of fatty acids and a substituted phosphoric acid group attached to a choline radical. Only 2% of the total choline in beef liver is in the free form (34).

The concentration of choline in animal tissue is proportional to their phospholipid content. Nerve tissue, liver, kidneys, brain, heart, and egg yolk are good sources (34).

III. Metabolism - Excretion

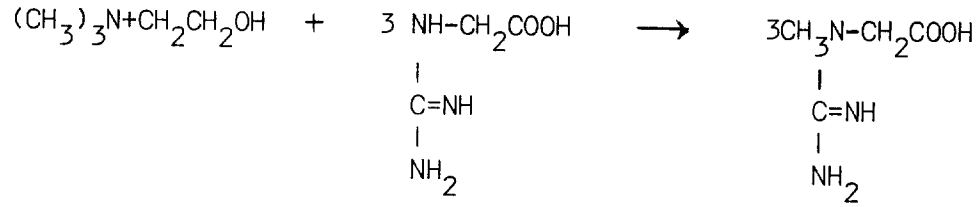
The most important physiological aspect of choline may be its role in transmethylation. The methyl groups attached to the quaternary nitrogen of choline are quite labile. One notable transmethylation involves the synthesis of methione, and essential amino acid (81).



Choline Homocysteine Methionine Ethanolamine

Choline may be synthesized in vivo by reversing this reaction, using either methionine or betaine as the methyl donor.

Choline transmethylation is important in the synthesis of creatine, as well (34).



Choline Guanidoacetic Acid Creatine

Choline can be replaced in this reaction by methionine and betaine (81).

Choline undergoes transacetylation to form acetylcholine, a neurohormone. Acetylation occurs in the presence of free acetic acid, Coenzyme A (CoA), ATP, and choline acetylase. As acetylation is an endergonic process, ATP provides the free energy; acetyl-CoA is the actual acetylating agent. The reactions are as follows (4):

1) ATP + acetic → adenyl acetate + pyrophosphate

2) Adenyl acetate + CoA → AMP + acetyl CoA

3) $(CH_3)_3N^+CH_2CH_2OH + CH_3CO{=}CoA \longrightarrow (CH_3)_3N^+CH_2CH_2O{\cdot}OCCH_3 + CoA$

Choline Acetyl CoA Acetylcholine

To prevent excessive accumulation of acetylcholine, choline esterase catalyzes the hydrolysis of acetylcholine to choline and acetic acid (34).

Choline is metabolized by the mitochondria to produce phosphatidyl choline (lecithin) by the following pathway:

1) Choline + ATP → phosphorylcholine + ADP

2) Cytidine triphosphate + phosphorylcholine ⟶
Cytidine diphosphate - choline + PPi

3) Cytidine diphosphate - choline + 1,2-diacylglycerol ⟶
phosphatidyl choline (lecithin) + cytidine monophosphate

Phosphatidyl choline (lecithin) is one of the two major lipid components of most membranes in animal cells (58).

Kennedy and Weiss named the enzyme for reaction 2, phosphorylcholine cytidyl transferase. This enzyme is widely distributed in nature. It has been found in the liver, brain, heart, and kidney of the rat, in the liver of the guinea pig and hog, in several strains of yeast, and in the carrot root. Reaction 3 is catalyzed by phosphorylcholine

glyceride transferase. Magnesium or manganese ions are necessary for phosphorylcholine glyceride transferase activity (46).

After the oral administration of choline to man, de la Huerga and Popper determined that 0.3% of the given dose was excreted in the urine as choline. After administration of 2-8 g choline base to 4 normal subjects, 60-67% of the choline N was excreted as trimethylamines of which 95-97% was trimethylamine oxide. This percentage was independent of the amount administered. Relatively little was excreted before 6 hours and after 12 hours. In vitro studies demonstrated that human feces converted choline to trimethylamines. Suppression of intestinal flora by antimicrobial agents inhibited urinary excretion of the trimethylamines. It was stated that the concept of extensive bacterial transformation of choline into trimethylamines appeared strange since choline absorption should take place in the upper part of the small intestine while bacterial activity starts in the large intestine (21).

Dyer and Wood reported that certain members of the Enterobacteriaceae family produced trimethylamine from choline in in vitro studies. All strains of Proteus rettgeri, P. ichthyosmius, P. vulgaris, P. mirabilis and Shigella alkalescens were positive (24).

IV. Effects on Enzymes and Other Biochemical Parameters

Choline is essential for normal growth of the rat, chick, and dog, and for lactation in the rat. Dietary deficiency has been demonstrated to be responsible for paralysis in the hind legs of nursing rats, inhibition of egg production in hens, slipped tendon (perosis) in chicks and young turkeys, and fatty liver in rats, dogs, rabbits, and pigs. Choline deficiency in young rats produces an acute hemorrhagic lesion of the kidneys. Even after short exposure to this condition and subsequent restoration to an adequate diet, such rats have been reported later in life to develop hypertension with cardiac enlargement and persistent renal damage (34).

Because of its biochemical function and its distribution in foods, choline is usually considered to be one of the vitamin B group. But it lacks the specificity characteristic of the vitamins and is a structural component of fat and nerve tissue. It has never been reported as a cofactor for an enzyme system. Therefore, according to Hawk et al. choline is not a vitamin (34).

Hawk et al. stated that mixed diets of man contain 0.5 to 1.0 g of choline daily, a quantity which compares better to amino acids than to vitamins (34).

Rosenberg (81) and Hawk et al. (34) have commented in their texts on the lipotropic effect of choline. In the absence of dietary choline, nuetral fat and cholesterol esters accumulate in the liver. This trend is reversed when choline is added to the ration. However, when high cholesterol feeding has induced the fatty liver in rats, choline feeding will not relieve the symptom.

In studies on the relationships between the development of arteriosclerosis and lipid pattern of blood and liver, Yamaguchi added various lipotropic drugs to the diet of cockerels on high cholesterol feed. Each group, except the control, was fed a basal diet plus cholesterol (1 g/kg of body weight) in 5 ml of cottonseed oil. One of those groups of 15 cockerels, 3 months of age and weighing 1.0 to 1.2 kg was given an oral dose of 250 mg/kg choline chloride per day for 16 weeks. Dosages were adjusted weekly to correspond to the change in body weight. The amount of lipids in the blood was measured before the treatment and at 2-week intervals for the duration of the experiment. At autopsy the fat in the liver was measured immediately after the withdrawal of the blood. Choline chloride inhibited the increase of the lipid level in boood and liver and reduced the incidence of experimental arteriosclerosis (100).

Welch and Welch reported that additional dietary choline chloride exerted a lipotropic effect (preventing fatty infiltration of the liver) in mice on a high fat diet. The effect of measuring the amount of choline chloride added to the high fat diet was proportionate to an increase in the lipotropic effect only at levels less than 0.05% (97).

Ridout et al. reported that the feeding of 0.092% choline chloride to 6 groups of 10 Wistar rats (80-120 g) on a hypolipotropic diet decreased total liver lipids by 23.1% of the net liver weight. When 6 groups consumed 0.37% choline chloride, liver lipids decreased by another 1.1%. The percentage of total liver lipids for the basal, 0.092% and 0.37% diets were 31.8%, 8.7%, and 7.6% of the net weight respectively (79).

Twenty weanling Wistar rats were fed a basal diet plus 0.5% choline chloride for eight weeks; 20 rats consumed a basal diet only. The percent of fatty acids present in the liver was greatly reduced by choline chloride feeding. Feeding 1% Mazola oil in addition to 0.5% choline chloride enhances this effect (13).

Kubena reported that a level of 2645 mg per kg of choline chloride in the diet of Japanese quail (Coturnix c japonica) reduced the total quantity of body fat (53).

Dietary choline chloride was found to lower the cholesterol contents of liver and blood of albino rats (150 g) consuming a high cholesterol (1%) diet. Unusually large amounts of bile acids were excreted in the feces, with no increase in cholesterol excretion. Chakrabartic and Banerjee suggested that choline may increase the conversion of cholesterol to bile acids (16).

Female rats were fed a diet containing a fat mixture corresponding to the level of fat in an average American diet plus 0.1% or 0.3% choline chloride. The higher choline chloride level produced a lowered plasma cholesterol content (94).

Firstbrook fed 14 adult male rabbits of various breeds 1 g of cholesterol 6 days a week for 9 weeks. Five animals received 500 mg/kg (1 g) of choline chloride 6 days a week, in addition. Nine controls received empty capsules only. All consumed Purina Rabbit Chow Checkers

ad libitum. After 9 weeks, all animals were sacrificed and the entire aortas removed and fixed. While the group on cholesterol only, had high blood cholesterol levels and a high degree of aortic atherosclerosis, the choline chloride and control group had neither effect. The choline chloride rabbits grew normally (28).

V. Drug Interaction

No Information Available

VI. Consumer Exposure Information

Choline is used as a nutrient and dietary supplement in foods. The following tables were compiled from data submitted by user firms. Food consumption values for each food category were derived from the Market Research Corporation of America (MRCA) data on frequency of eating and from the USDA data on mean portion size of foods in each food category. The food consumption values thus derived were coupled with the usage level data obtained in the surveys to calculate the daily intake of each substance.

Table 2 reports the usage of choline salts and Table 3 their use in infant formulas or baby foods. Table 11 reports the annual poundage data for choline salts. Table 13 reports the possible daily intake per food category and total dietary based on food consumption by total sample. Table 14 reports potential daily intakes in mg of NAS Appendix A substances (Groups 1 and 11) per food category reported, based on food consumption by eaters only.

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/03/72

TABLE 11, PART A -- ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

SUBSTANCE NAME (SURVEY NO.)	# REPORTS TO NAS 1960/1970	POUNDAGE REPORTED TO NAS (MATCHING REPORTS FOR BOTH YEARS) 1960	1970	TOTAL 1970 POUNDAGE REPORTED TO NAS	# REPORTS TO FEMA	POUNDAGE REPORTED TO FEMA-- 1970 ONLY	TOTAL 1970 POUNDAGE NAS + FEMA
CHOLINE BITARTRATE NAS 0065	*/ *	40,400	37,069	37,069			37,069
CHOLINE CHLORIDE NAS 0066	5/ 5	4,000	9,137	9,137			9,137

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/02/72

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
CHOLINE BITARTRATE NAS 0065	05 MILK PRODS(R)	*	0-5 MO.	2.322000	1.720000	2.322000
			6-11 MO.	26.832000	129.643000	26.832000
			12-23 MO.	23.435000	74.992000	23.435000
			2-65+ YR.	16.985000	51.858000	16.985000
CHOLINE BITARTRATE NAS 0065	83 FORMULAS(B)	*	0-5 MO.	379.341000	694.950000	379.341000
			6-11 MO.	77.292000	368.267000	77.292000
			12-23 MO.	24.860000	7.006000	24.860000
CHOLINE BITARTRATE NAS 0065	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	381.663000	696.670000	381.663000
			6-11 MO.	104.124000	497.310000	104.124000
			12-23 MO.	48.295000	81.998000	48.295000
			2-65+ YR.	16.985000	51.858000	16.985000
CHOLINE CHLORIDE NAS 0066	83 FORMULAS(B)	5	0-5 MO.	32.260770	59.101500	37.598400
			6-11 MO.	6.573240	31.318990	7.660800
			12-23 MO.	2.114200	.595820	2.464000
CHOLINE CHLORIDE NAS 0066	ALL CATEGORIES ********** **********	5	0-5 MO.	32.260770	59.101500	37.598400
			6-11 MO.	6.573240	31.318990	7.660800
			12-23 MO.	2.114200	.595820	2.464000

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/01/72

TABLE 2 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
CHOLINE BITARTRATE NAS 0065	05 MILK PRODS(R)	*	.04300	.04300
CHONDRUS EXTRACT NAS 0067 FEMA 2596	01 BAKED GOODS(R)	*	.06100	.26100
	05 MILK PRODS(R)	11	.05322	.09594
	06 CHEESE(R)	*	.00500	.00600

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/02/72

TABLE 3 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP II) USED IN INFANT FORMULA PRODUCTS & BABY FOODS(B)

NAS SURVEY NO. SUBSTANCE NAME	FOOD CATEGORY NO. NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
0065 CHOLINE BITARTRATE	83 FORMULAS(B)	*	.11300	.11300
0069 CHOLINE CHLORIDE	83 FORMULAS(B)	5	.00961	.01120

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ESSENTIAL FATTY ACIDS AND LIPOTROPIC ACTION OF INOSITOL

OVER a year ago the author became interested in the possible effect of essential fatty acids on the lipotropic action of choline. If choline lowers fat in the liver by virtue of its incorporation into the lecithin molecule, then essential fatty acids, which also constitute integral parts of this phospholipid, might similarly be required before choline can exert this effect. A preliminary experiment of three weeks' duration, using rats 100 to 125 grams in weight, suggested that the essential fatty acids were not required for the action of choline, although the results obtained did suggest that choline decreases liver fat to a greater extent when these metabolites are present. However, the difference found was of questionable statistical significance. It was realized that the three-week period would not have permitted a marked depletion of the animals' stores of essential fatty acids, and an experiment of longer duration was contemplated.

In the meantime Engel's paper[1] on the relation of the essential fatty acids to the lipotropic action of choline was published. In an experiment of eight weeks' duration performed on weanling rats, Engel found that pyridoxine was required for the full lipotropic action of choline. Because of the relationship of pyridoxine and essential fatty acids in the cure of rat acrodynia, Engel determined under similar conditions the effect of essential fatty acids on the lipotropic action of choline. He found that they also augmented the lipotropic action of this substance.

[1] R. W. Engel, *Jour. Nutrition*, 24: 175, 1942.

Science 99(2583):539-540. 1944

An experiment of eight weeks' duration was devised to test the effect of essential fatty acids upon the lipotropic action of choline and also of inositol since it, too, is a constituent of certain phospholipids and its influence might be similarly affected by essential fatty acids in the diet. The basal diet chosen for this experiment consisted of 8 per cent. casein, 12 per cent. casein, 12 per cent. gelatin (both extracted with 1:1 alcohol-ether), 73 per cent. sucrose, 5 per cent. salt mixture, 2 per cent. agar, 0.015 per cent. vitamins A and D concentrate (Ayerst, McKenna and Harrison, containing 500,000 I.U. of A per gram and 50,000 I.U. of D per gram). A mixture of the B vitamins in the following amounts was injected daily in 0.5 ml physiological saline: thiamine chloride, 50 α, riboflavin, 25 α, pyridoxine, 20 α, calcium pantothenate, 100 α, nicotinic acid, 100 α. Twenty weanling rats of the Wistar strain (23 to 35 days old) were used for each group and litters were divided as evenly as possible amongst the different groups.[2] The groups were also balanced with respect to weight and sex. After eight weeks on the diet, the rats were killed by a blow on the head. Individual liver fats were determined in the usual way by saponification, acidification and extraction of the fatty acids with petroleum ether. Table 1 shows the results obtained.

This result supports Engel's statement that the lipotropic action of choline is increased in the presence of Mazola oil presumably through the action of essential fatty acids[1]. The lipotropic action of inositol on the other hand was obliterated by the inclusion of Mazola oil.[3] A possible explanation of this phenomenon might be that certain fatty acids in this oil make the diet more nearly adequate, increasing the demand for lipotropic factors and thus promoting a greater deposition of fat in the liver. But in view of the results with choline the writer believes that one must look elsewhere for the true explanation.

[2] The mortality of the rats on the choline-free diets was quite high because of the development of hemorrhagic kidneys.

TABLE 1

EFFECT OF ESSENTIAL FATTY ACIDS ON LIPOTROPIC ACTION OF CHOLINE AND INOSITOL

Diet No.	Supplement per cent. of diet	Fatty acids per cent. liver weight
1. (Basal)	.....	23.4
2.	Choline chloride (0.5)	6.43
3.	Inositol (0.3)	13.3
4.	Mazola Oil (1.0)	25.3
5.	Mazola Oil (1.0) + Inositol (0.3)	27.2
6.	Mazola Oil (1.0) + Choline (0.5)	4.71

It is more probable that the nature of the fatty liver is changed in the presence or absence of the various supplements used. It will be recalled that choline has a relatively greater lipotropic effect on the "fat" fatty liver than on the "cholesterol" type of fatty liver, whereas with inositol, the reverse is true.[4] Only a complete analysis of the liver fats would reveal whether or not such a hypothesis is tenable. Fractionation of the fats from the livers of rats fed diets identical with those described above is now in progress and the results will be published shortly.

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EFFECT OF CHOLINE AND RIBOFLAVIN ON FECAL EXCRETION OF CHOLESTEROL AND BILE ACID OF ALBINO RATS KEPT ON HIGH CHOLESTEROL DIET

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Kesten and silbowitz (1) noted that the level of plasma cholesterol was decreased in rabbits fed high levels of lecithin together with cholesterol. Lecithin has also been reported to lower serum cholesterol levels in man (2). It has been shown by Best *et al.* (3) that the active component of lecithin is choline. Kritchevsky *et al.* (4) have observed a lowering of serum cholesterol in rabbits after the injection of choline citrate. Harrill *et al.* (5) observed a lowering of cholesterol contents in the liver of albino rats fed a high fat diet containing high level of riboflavin. This method of decreasing cholesterol is of considerable interest in the study of arteriosclerosis and deserve further study. Since catabolism of cholesterol consists primarily in the conversion of bile acid (6) and appreciable amount of cholesterol is excreted in the feces, it was thought worthwhile to investigate whether or not choline and riboflavin will influence the fecal excretion of bile acid and cholesterol of animals kept on high cholesterol diet.

EXPERIMENTAL

Forty eight albino rats weighing between 120-150 g were distributed in four groups of twelve each. Groups 1, 2, 3 and 4 were fed *ad libitum* one of the 4 experimental diets shown in Table 1. The animals were sacrificed after 2,8 and 12 weeks of experiment by stunning. The liver lipids were extracted with acetone alcohol mixture (1 : 1). Cholesterol content of the liver, blood and feces was estimated by the method of Schoenheimer and Sperry (7). Fecal bile acid was estimated by the method of Snell (8).

RESULTS AND DISCUSSION

Data represented in Tables 2 and 3 indicate clearly that both riboflavin and choline when given in high doses along with high cholesterol diet lower the cholesterol contents of liver and blood of albinorats. Choline showed a more pronounced cholesterol lowering effect in liver than riboflavin. The animals receiving a high dose of riboflavin along with 1 % cholesterol containing the basal diet did

TABLE 1
Composition of Experimental diets

	Group			
	1	2	3	4
	per cent			
Casein	20	20	20	20
Sucrose	65	64	64	64
Groundnutoil	10	10	10	10
Salt mixture [a]	4	4	4	4
Vitaminised sucrose [b]	1	1	1	1
Cholesterol	0	1	1	1
Choline chloride (mg) [c]	0	0	0	1000
Riboflavin (mg) [d]	0	0	50	0

[a] Hawk-Oser salt mixture.
[b] One gram of vitaminised sucrose contains the following: thiamine HCl 500 μg; riboflavin 250 μg; pyridoxine 200 μg; calcium pantothenate 1,000 μg; niacin 1,0[illegible] g; cyanocobalamin 1 μg; folic acid 100 μg; choline 120 mg; α-tocopherol acetate 2.2 mg; menadione sodium bisulphite 100 μg; biotin 10 μg; vitamin A 1100 USP units, calciferol 200 I.U.; inositol 10 mg, made up to 1 g with sucrose.
[c] Added in addition to the normal amount supplied by the vitaminised sucrose.
[d] Added in addition to the normal amount supplied by the vitaminised sucrose.

TABLE 2
Effect of Choline and Riboflavin on Liver and Blood Cholesterol of Albino Rats Fed high Cholesterol diet

No. of animals.	Group [a]	Experimental period	Average total cholesterol contents	Total blood cholesterol
		weeks	*mg/100 g liver*	*mg/100 ml*
3	1	0 (Initial)	266±12.5 [b]	48.6±5.2
3	2	2	247±15.7	56.4±2.2
3	2	8	206±10.2	40.0±6.5
3	2	12	253±10.5	42.6±8.2
4	2	2	1298± 98.8	70.2± 8.1
4	2	8	1888±185.5	86.6±10.3
4	2	12	2164±210.5	87.6±12.5
4	3	2	1610±150.5	70.7±5.9
4	3	8	1710±110.8	65.0±7.1
4	3	12	1700±145.5	61.0±9.1
4	4	2	1200± 89.5	65.0±6.2
4	4	8	1420± 98.2	52.0±3.1
4	4	12	1300±100.8	52.0±5.3

[a] Desccribed in Table 1 [b] Standard deviation.

not show any appreciable increase in cholesterol and bile acid excretion in feces. The animals receiving a high dose of choline along with 1% cholesterol containing basal diet excreted large amounts of bile acids in feces without showing any increased excretion of cholesterol in feces. The cholesterol lowering effect of choline observed in this experiment may be due to the excess degradation of cholesterol to bile acid which is excreted in the feces.

Mannering, Lipton and Elvehjem (9) observed that symptoms of riboflavin

TABLE 3

Effect of Choline and Riboflavin on daily fecal excretion of Cholesterol and bile acid of albino Rats Fed high cholesterol diet

No. of animals	Experimental period	Group[a]	Daily fecal excretion	
			Cholesterol	Bile acid
	weeks		*mg per rat*	
4	12	1	8.4±2.7[b]	11.8±1.5
4	12	2	17.8±5.2	33.6±3.1
4	12	3	18.5±2.1	31.8±4.8
4	12	4	16.8±6.2	50.5±4.5

[a] Described in Table 1 [b] Standard deviation

TABLE 4

Food Intake and Weight gain

Group[a]	No. of animals	Average daily food intake/rat during the experimental period			Average weight gain/rat during the experimental period			
		Week 2	Week 8	Week 12	Initial	Week 2	Week 8	Week 12
1	4	11.2±1.8[b]	12.5±2.2	15.2±1.8	134±7.5	165.1±10.5	185.4±12.0	216.5±14.8
2	4	10.8±2.2	12.1±2.8	14.2±2.1	136±5.4	170.4±11.2	181.9± 8.5	196.8±10.2
3	4	11.4±3.2	13.5±1.2	14.8±2.2	136±5.8	171.4± 8.2	195.8±10.1	238.4±12.8
4	4	11.8±2.3	12.8±2.1	15.4±2.4	135±6.4	168.2± 9.8	198.1± 8.2	240.8±11.8

[a] Described in Table 1 [b] Standard deviaton

deficiency in rats increase in severity upon administration of increased amounts of fats. They have further observed that when increased doses of riboflavin are given together with increased amounts of fats, no deleterious effect is observed. Bruggen, Hutchens and West (10) fed randomly labeled cholesterol to rats and found an appreciable amount of administered label in fatty acids. Kritchevsky *et al.* (11) observed the conversion of cholesterol to fatty acids with tritium labeled cholesterol. These experiments suggest that one hitherto uninvestigated pathway of cholesterol catabolism involves conversion to fatty acids. Mahler (12) has shown that riboflavin functions as a part of butyryl coenzyme A dehydrogenase which is essential to fatty acid metabolism. Thus it seems likely that a high cholesterol diet increases the requirement of riboflavin for the proper utilisation of cholesterol and when an increased dose of riboflavin is given together with increased amounts of cholesterol, less deleterious effect is observed.

SUMMARY

The influence of high dose of choline and riboflavin on the fecal excretion of cholesterol and bile acids of albino rats kept on a high cholesterol diet was studied. Choline increased the fecal excretion of bile acid without showing any appreciable increase in the excretion of cholesterol in feces. Riboflavin did not show any appreciable increase or decrease in the excretion of cholesterol and bile acid in the feces of the animals fed a high cholesterol diet. Both choline and riboflavin exerted a cholesterol-lowering effect in the liver and blood of the animals fed a high cholesterol diet.

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THE EXPERIMENTAL PRODUCTION OF A HYPERCHROMIC ANEMIA IN DOGS WHICH IS RESPONSIVE TO ANTI-PERNICIOUS ANEMIA TREATMENT[1]

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The author has reported recently (1) that the daily administration of choline chloride to normal dogs produced a significant depression of their red blood cell counts within 10 to 15 days, presumably by decreasing the rate of erythropoiesis.

This paper proposes to report an accentuation of this anemia by the administration of more than one dose of choline daily, and the response of this anemia to liver injection U.S.P. and stomach U.S.P.

PROCEDURE. Seven dogs were maintained on a diet of Purina Dog Chow and rolled oats, with water ad libitum. This diet is adequate in the sense that dogs remain healthy and maintain or gain weight, even when kept on it for many months. Two of our dogs have thrived on this diet in our laboratory for over two years.

Red blood cell counts and hemoglobin percentages (Hellige) were determined frequently, and total leukocyte counts, hematocrit readings (Van Allen) and reticulocyte percentages were determined at least occasionally. Blood samples for these determinations were drawn from the external saphenous veins of the dogs while they were lying upon a table, blindfolded, in an unexcited and fairly basal condition, at least 7 (and usually 11) hours after any previous administration of drugs or food.

To produce the anemia, four of the dogs were given one dose of choline hydrochloride[2] daily (10 mgm. per kgm) by stomach tube at the start of the experiment and for at least 25 days. Then after a definite anemia had been established, they were put on two daily doses, each of 10 mgm. per kgm. of body weight. After a lower red cell level had been reached on this regime, a third daily dose was added to each dog's program. The single doses were kept at constant magnitude (i.e., 10 mgm. per kgm.) and they were spaced about 6 or 7 hours apart during the day. Although the program was occasionally varied slightly, doses were given usually at 9 a.m., 4 p.m. and 11 p.m. daily, including Sundays and holidays. One other dog was "Accelerated" by being put on 2 daily doses of choline 7 days after the start of medication, and on 3 daily doses beginning on the 26th day of medication. Two other dogs, who were new and not well acclimated to this laboratory, were given 3 daily doses of choline from the outset of their experiments and continued thereon.

Liver injection, U.S.P., was administered to three dogs after their anemias had reached an apparent maximum. It consisted chiefly of the daily intramuscular injection of 2 cc. of a purified solution of liver containing U.S.P. unit per cc.[3] One dog, however, received 0.5 cc. of a solution containing 15 U.S.P. units per cc., daily for 7 days. Choline feeding was continued at the regular rate throughout the periods of liver administration.

Stomach U.S.P. (Ventriculin[4]) was fed to one dog in daily doses of 20 grams, in addition to choline, after his anemia had been established.

Atropine sulfate (0.5 mgm. per kgm.) was given in dilute solution by stomach tube to one anemic dog 3 times daily, in addition to the three daily doses of choline.

RESULTS. Figure 1 shows the red blood cell counts of 5 dogs during the development of their anemias by choline feeding. It will be seen that the erythrocyte numbers of these dogs were reduced finally by 30 to 43 per cent, due to choline administration. Hemoglobin percentages were reduced, *but not so greatly*, and indeed not proportionately. Table 1 shows the status of the color index at different levels of the red cell counts in 2 dogs. For the purpose of computing the color index, the author has arbitrarily placed the dog's normal red blood cell count at 6 million, and the normal value for 100 per cent hemoglobin at 13 grams per 100 cc. of blood (or 90 per cent on the Wintrobe scale). This was done after inspecting data in articles by Wintrobe (2) and Van Loon and Clark (3) and also data assembled by this author. This anemia may be classed as macrocytic in type, since we found relative volume indices of 1.18 and 1.45 in 2 dogs in which it was calculated. Total leukocyte counts did not change much although they were observed to be diminished by about 12 per cent in three dogs. Reticulocyte percentages during the anemia varied from about 0.05 per cent to 0.6 per cent.

Gastric juice containing free hydrochloric acid was obtained from two dogs after an alcohol test meal or histamine injection. In three others no significant amount of juice was obtained, but this may have been due to imperfect technique.

Figure 2 shows the response of the anemia to purified solution of liver, which was injected intramuscularly into 3 dogs (long dashes, fig. 2). Two of the animals received 2 units daily for about 27 days, and they responded by regenerating at least 85 per cent of their red blood cell deficits during this time in spite of continued choline feeding. Hemoglobin regeneration lagged behind the erythrocyte production. One dog (fig. 2) served as a control for 3 weeks, and was then given a potent liver solution (15 units per cc.) in the dosage of 7½ units per day, intramuscularly. He responded with a rapid rise in red cell count within the 7 days during which he was observed. Reticulocyte percentages arose to only 1.5 to 2.3 in these dogs during liver therapy. The sixth day of therapy showed peak reticulocytosis.

One dog was given atropine sulfate 3 times daily in addition to regular doses of choline. As will be seen in figure 2 (short dashes), this animal showed a red blood cell count increase that very closely resembled those obtained in the liver treated dogs. Hemoglobin percentage increased, but not as greatly or as fast

[1] Research paper no. 551, journal series, University of Arkansas.

[2] Part of the choline chloride used in these experiments was a gift from the Lederle Laboratories, New York, New York.

[3] "Campolon" was generously supplied by Winthrop Chemical Co., New York, New York.

[4] Ventriculin was kindly supplied by Parke-Davis & Co., Detroit, Michigan.

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as the red cell number. The reticulocyte percentage reached only 1.1 per cent on the 8th day of atropine administration.

Ventriculin was fed one anemic dog in a daily dose of 20 grams for 12 days. In this time his erythrocyte number and hemoglobin percentage returned to its normal value, in spite of continued choline administration. On the 7th day of therapy, his reticulocyte percentage was determined to be 2.4.

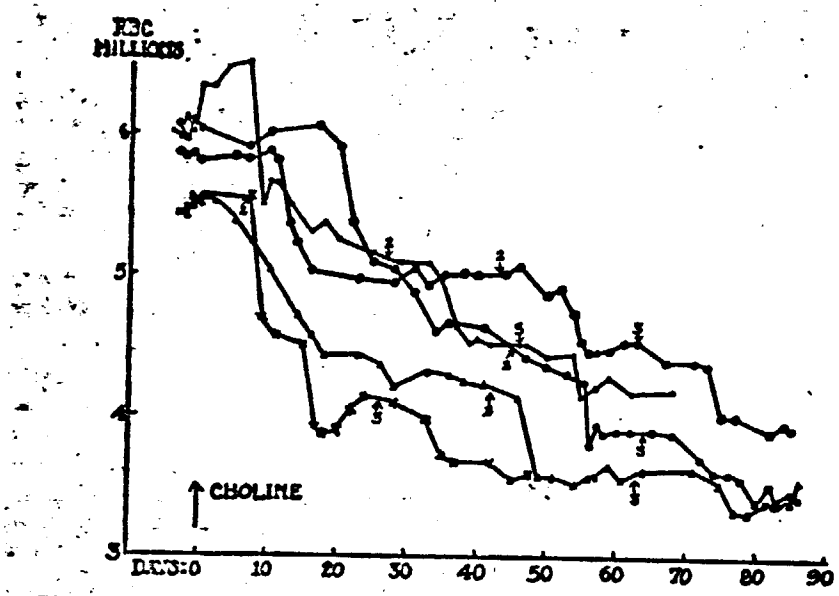

Fig. 1. Red blood cell counts obtained on five dogs during the development of choline anemia. *2* indicates commencement of the administration of two doses daily of choline. *3* indicates start of 3 doses daily for each dog as indicated.

TABLE 1

Color index changes in two anemic dogs treated by liver injection

	Dog 4			Dog 9		
	RBC millions	Hemoglobin per cent	C.I.	RBC millions	Hemoglobin per cent	C.I.
Normal control values	6.04	90	1.01	5.50	85	1.03
During anemia	3.51	64	1.22	3.50	58	1.10
During liver therapy	4.50	72	1.06	4.45	64	0.96
	5.10	75	0.98	4.75	66	0.92
	5.50	80	0.97	5.00	68	0.91

Discussion. It appears in figure 1 that the use of more than one dose of choline daily augments the anemia that is produced by one dose daily. We have taken one anemic dog which had been receiving 3 daily doses of 100 mgm. each, and put him on a schedule in which he received only one large dose of 600 mgm. daily (not shown). He responded with a rise in his red cell count after about one week. Furthermore we have previously found that doubling the single daily dose of choline did not increase its action in reducing experimental polycythemia. These facts lead us to believe that the magnitude of the response to choline is a function of the time of exposure to its action.

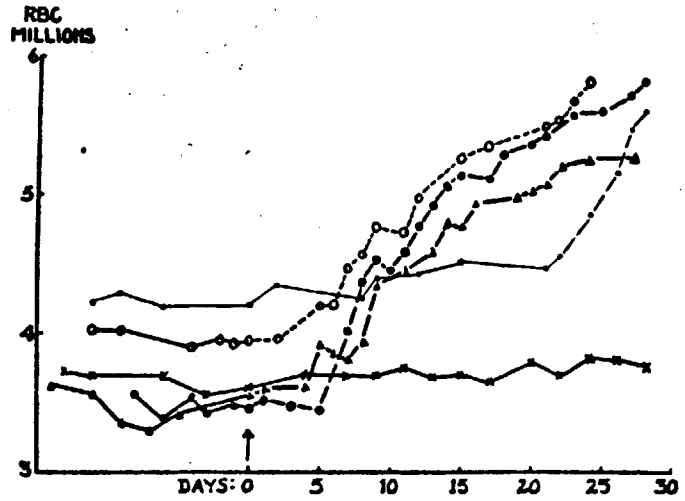

Fig. 2. The effect of liver injection or atropine upon anemic dogs. Long dashes indicate periods of liver administration in 3 dogs. Short dashes indicate simultaneous administration of atropine sulfate with choline in one dog.

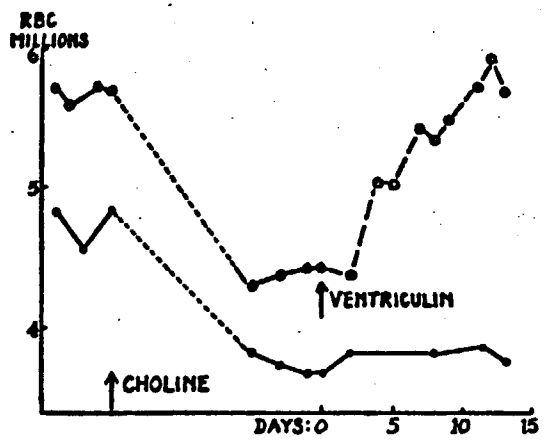

Fig. 3. The effect of stomach U.S.P. on the erythrocyte number of one anemic dog.

We believe, as we have stated previously (1, 4), that choline depresses erythropoiesis by increasing the blood and oxygen supply to bone marrow, through its vasodilator action. Since atropine antagonises the action of choline in these experiments (fig. 2) and in experiments reported previously (4) the action of the latter must be a muscarinic action. We believe that the important action must be *vasodilatation*, especially in view of our previous experiments (4, 5) in which

other vasodilator drugs such as nitrites, aminophylline and certain choline esters and ethers were shown to be effective in reducing experimental polycythemias.

We cannot say that it is the anti-pernicious anemia principle that causes the regeneration of red blood cells in these experiments, although such a conclusion is suggested by the fact that two brands of purified solution of liver, as well as ventriculin, were effective in returning the erythrocyte numbers virtually to normal.

We do not know the mechanism by which liver and stomach, U.S.P., cause a remission of this "choline anemia." The remission resembles superficially, at least, that induced by atropine in one dog of our series. In acute experiments we have been unable to find evidence for any atropine-like action or any circulatory action in highly potent purified solutions of liver. We were unable to find any evidence for the presence of choline oxidase in the liver solutions, as was perhaps to be expected since this enzyme is not very extractable from liver (6). It seems quite possible to the author that liver injection may contain or form a synthesizing enzyme which removes free choline.

The possibility exists that dogs with choline-induced anemia could be used for the biological assay of anti-pernicious anemia preparations.

It should be pointed out, however, that great care must be taken to draw blood samples only when the animals are unexcited and in a resting state, if experiments of this sort are to be successfully conducted.

CONCLUSIONS

The oral administration of 3 doses daily of choline chloride (10 mgm. per kgm. each) to 5 dogs produced reductions of from 30 to 43 per cent in their red blood cell counts. Milder anemias were produced in 2 other animals. The anemias, so produced, are hyperchromic and probably are the result of a depression of erythropoiesis.

The intramuscular injection of adequate doses of purified solutions of liver into 3 anemic dogs caused their erythrocyte numbers to respond with increases which were marked by the 7th day, and (in two animals) approximated normal after 4 weeks. These responses occurred in spite of continued choline administration.

Stomach, U.S.P., fed in daily doses of 20 grams to one mildly anemic dog, caused a return of his red cell number and hemoglobin percentage to normal within 12 days, in spite of continued choline feeding.

The oral administration of atropine sulfate 3 times daily to one anemic dog caused his red cell count to return to normal in 4 weeks in spite of continued choline feeding.

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URINARY EXCRETION OF CHOLINE METABOLITES FOLLOWING CHOLINE ADMINISTRATION IN NORMALS AND PATIENTS WITH HEPATOBILIARY DISEASES[1]

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Choline has been recommended for the treatment of hepatic diseases, especially those of nutritional origin, and for the prevention of arteriosclerosis. Little is known of its fate after oral or intravenous administration. A recent Spanish report (1) that patients with liver diseases and dogs with experimental hepatic damage excrete in the urine considerably more than normal of a substance precipitated by Reinecke salt (and considered as choline by the authors) after large doses of choline given orally, suggested that the utilization of choline might be altered in hepatobiliary disease. In addition, studies with radiophosphorous (2) indicate a reduced rate of phospholipid formation in the presence of hepatocellular damage and an increased rate in biliary obstruction, suggesting that less choline might appear in the urine than normal in obstructive jaundice and more than normal in liver cell damage.

To test these hypotheses and to investigate the fate of choline after its oral administration *1)* the identity of the reineckate precipitated from the urine was determined, and *2)* the influence upon its excretion of physiologic conditions as well as hepatobiliary diseases was studied.

MATERIAL

Four normal subjects and 22 patients with various hepatobiliary diseases were given on separate occasions single doses of choline bicarbonate[3] equivalent to 2-8 grams of choline base (230 920 mg. choline N). Their diets were not controlled. Urine samples, collected at various intervals, were preserved with 25 ml. of concentrated hydrochloric acid (diluted with equal parts of water) per liter of urine.

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EXPERIMENTAL

Chemical identification of the reineckate obtained from urines after choline administration and procedures applied for quantitative determination of the metabolites in the urine

Reagents used:

1) Three per cent solution of Reinecke salt, freshly prepared and filtered.
2) Aqueous saturated solution of sodium hydroxide.
3) Aqueous saturated solution of potassium carbonate.
4) Folin and Ciocalteu reagent (3), diluted with three volumes of water before use.
5) Ten per cent aqueous solution of anhydrous sodium carbonate.
6) Choline reineckate: to 40 ml. of 1 per cent choline chloride 20 ml. of the Reinecke salt solution are added. After centrifugation the precipitate is washed three times with 20 ml. portions of water. The crystals are then dried at 37° C. and stored. Immediately before use a saturated solution is freshly prepared by adding some of the choline reineckate to 0.1 normal sodium hydroxide, shaking and filtering.
7) Acetone, Norit A, Devarda's alloy, octyl alcohol, concentrated and 0.5 normal hydrochloric acid, normal and 0.1 normal sodium hydroxide.

The alkali-insoluble reineckate: The reineckate precipitated at a pH of 6.5 from urines after administration of choline (following the method of the Spanish authors [1]), was almost completely soluble in 0.1 normal sodium hydroxide. Since choline reineckate was found to be hardly soluble in this solution, the great bulk of the precipitate was not choline reineckate. On nitrogen basis, only 0.7 mg. of choline reineckate is soluble in 100 ml. of 0.1 normal sodium hydroxide while 113 mg. of urinary acid-precipitable reineckate are soluble in this solution. To determine the traces of alkali-precipitable reineckate (choline) the following procedure was used, adapted from the method of Glick (4).

Determination of choline: Saturated sodium hydroxide is added to 70 ml. of urine until a blue color to thymolphthalein appears. The precipitate is removed by filtration and to an aliquot, representing 50 ml. of urine, 25 ml. of Reinecke salt solution are added. The Reinecke precipitate formed in four hours in the refrigerator is spun

TABLE I

Recovery of choline added to urine with the method used

Diagnosis	Mg. choline N added to 100 ml.	Mg. choline N found per 100 ml.	Per cent choline N recovered
Normal	0.250	0.240	96
	0.750	0.710	93
	1.250	1.280	102
Hepatitis	0.250	0.220	88
	0.750	0.740	98
	1.250	1.360	107

down. It is washed with the saturated solution of choline reineckate in order to remove any co-precipitated non-choline reineckate. The final residue is dissolved in acetone containing 1 per cent glacial acetic acid. The solution is filtered if necessary and made to a noted volume. The transmittancy is read in a spectrophotometer or colorimeter at a wave length of 520 mμ. A standard curve is made with choline chloride using the same procedure. The results are calculated in mg. of choline N per ml. of the acetone reineckate solution. Choline added to urines from normal or hepatitis patients was recovered quantitatively (Table I). The amount demonstrated by this method represented in the studied cases 0.22 to 4.40 per cent of the total reineckate in the 24-hour urine and 0.27 to 1.04 per cent of the total amount of the choline N ingested.

The alkali-soluble reineckate: To demonstrate whether the alkali-soluble reineckate represents one of the recognized metabolites of choline such as betaine, dimethylamine or trimethylamine or its oxide, the reineckate precipitated from 10 ml. of acidified urine from normals and from patients suffering from hepatic diseases after administration of choline was dissolved in 20 ml. of 50 per cent aqueous acetone. Then 5 ml. of aqueous saturated silver sulfate solution were added (5) to remove the Reinecke ion. In the filtrate, the excess silver was removed with 5 ml. of normal hydrochloric acid and the water clear filtrate was evaporated under partial vacuum and under nitrogen at 30° C. The residue was dissolved in 10 ml. of methyl alcohol, filtered and evaporated as above. The final residue was dissolved in 20 ml. of water. An aliquot (10 ml.) was alkalinized with potassium carbonate and aerated for two hours at room temperature in the apparatus described by Sobel and associates (6) into 5 ml. of 0.5 normal hydrochloric acid. To this, 3 ml. of Reinecke salt solution were added and the crystalline precipitate obtained showed the microscopic appearance and melting point (275° C.) characteristic of trimethylamine reineckate. Mixing of the crystalline precipitate with trimethylamine reineckate did not alter the melting point. The melting point of the picrate was 216° C., identical with that of trimethylamine picrate and was also not altered by mixing with trimethylamine picrate.

To investigate whether trimethylamine oxide (TMAO) was also present in addition to trimethylamine (TMA) (the former not being aerated under the experimental conditions used), another aliquot of 10 ml. was acidified with hydrochloric acid to normality. It was then reduced with 0.5 gram of Devarda's alloy and placed in a water bath at 90° to 95° C. for 40 minutes. After centrifugation the supernatant was transferred to the aerating apparatus and enough saturated sodium hydroxide was added to redissolve the precipitate formed and then 3 ml. of saturated potassium carbonate were added. The mixture was aerated as before. In four experiments (two normal subjects and two patients with cirrhosis), the TMAO accounted for 90 to 97 per cent of the total reineckate, TMA for 5 to 10 per cent and choline for 0.56 to 0.77 per cent.

Determination of total trimethylamines (TTMA) with Reinecke salt: To 50 ml. of acid urine about 2 grams of Norit A are added and the mixture shaken and allowed

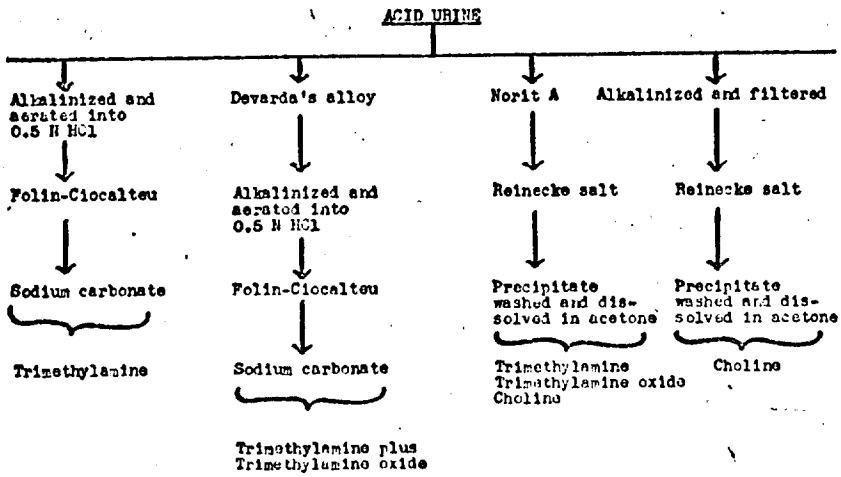

FIG. 1. ANALYTICAL SCHEME FOR THE QUANTITATIVE DETERMINATION OF CHOLINE, TRIMETHYLAMINE AND TRIMETHYLAMINE OXIDE

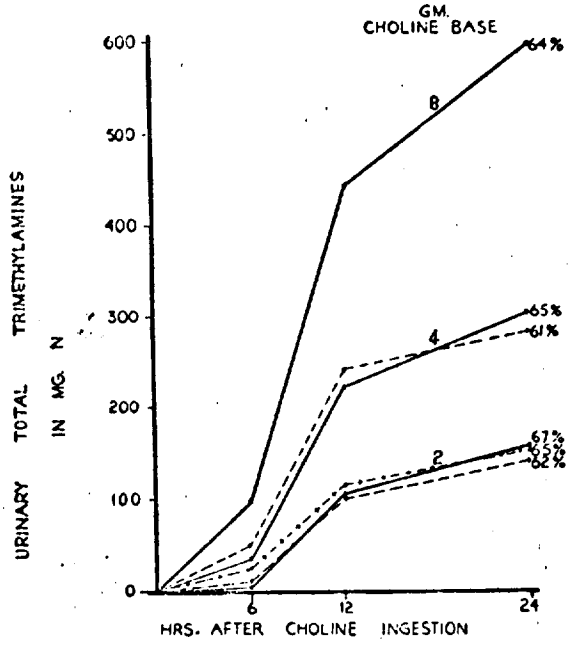

FIG. 2. URINARY EXCRETION OF TRIMETHYLAMINES BY NORMAL PERSONS AFTER ORAL ADMINISTRATION OF VARIOUS AMOUNTS OF CHOLINE

The percentage refers to the amount of trimethylamine N in relation to choline N administered. Each type of line refers to the same experimental subject.

to stand for at least 30 minutes. In some concentrated urines it is necessary to dilute the Norit filtrate. To 5 or 10 ml. of the filtrate, half a volume of the Reinecke salt solution is added. The crystalline precipitate obtained after standing at 10° C. for one hour is collected onto a fine sintered glass filter using vacuum and washed with saturated aqueous solution of "urine acid-precipitable reineckate," prepared from the same urine. The error produced by using a saturated solution of the reineckate does not exceed 20 micrograms and is, therefore, negligible. The crystals are then dissolved in acetone. The color is read in the spectrophotometer as in the determination of choline. The extinction co-efficient of the Reinecke salt of TMAO (prepared according to Dunstand and Goulding [7]), was found to be the same as that of choline, betaine and TMA chlorides when expressed as nitrogen. The very small amount of choline determined with TTMA can be ignored. The presence of betaine or other quaternary ammonium bases was excluded because the reineckate precipitable material was shown to be quantitatively removed by reduction and aeration.

Use of Folin-Ciocalteu reagent: The quantitative relation between TMA and TMAO was investigated by determining with the Folin-Ciocalteu reagent the TMA in the aerates from alkalinized urines with and without previous reduction. This reagent, otherwise used for the determination of phenols, gives a blue color with TMA (8) but not with ammonium, mono- or dimethylamine salts. Since the phenols of the urine are not aerated under the experimental conditions, the color reaction can be considered specific for TMA. This offers an alternative and simple method for the determination of TTMA even if present in small amounts. This method which has been used in this study for the determination in the stool cultures has subsequently been proven applicable for urine specimens.

Method for determination of trimethylamines with the Folin-Ciocalteu reagent: For determination of TMA, 10 ml. of urine are neutralized with saturated sodium hydroxide, followed by addition of 3 ml. of saturated potassium carbonate and 4 or 5 drops of octyl alcohol. The mixture is aerated and the TMA is collected as described before. An aliquot is neutralized with normal sodium hydroxide and made to 5 ml. with water; 2 ml. of Folin-Ciocalteu reagent and 3 ml. of the sodium carbonate solutions are added. After 15 minutes the developed color is read in a spectrophotometer at 620 mμ. A standard curve is made with pure, dried TMA hydrochloride. For determination of TTMA, 10 ml. of urine are mixed with enough concentrated hydrochloric acid to make it normal. The sample is treated with Devarda's alloy as previously described, to reduce TMAO to TMA (9). After cooling, saturated sodium hydroxide solution is added until the precipitate formed re-dissolves. Now 3 ml. of saturated po-

TABLE II

Urinary excretion of choline and trimethylamines in normal individuals within 24 hours after administration of 2 grams choline base

Choline		Total trimethylamines			
Mg. N	Per cent of choline N fed	Mg. N	Per cent of choline N fed	Per cent excreted in first 12 hours	(Choline/TMA) X100
1.20	0.53	155.0	67.0	67.5	0.77
0.80	0.35	112.3	61.8	70.2	0.56
0.90	0.39	150.0	65.0	77.0	0.60
0.75	0.33	138.2	60.0	65.5	0.54

tassium carbonate are added and the sample is aerated as before. The aerate is conveniently diluted and treated with the Folin-Ciocalteu reagent. TMAO is calculated from the difference between the values before and after reduction. Choline and betaine are not converted into TMA and, therefore, fail to give the reaction.

The principles of the procedures applied are illustrated in Figure 1.

Variations of urinary choline and TTMA excretion under physiologic conditions

Excretion under basal conditions: In four normal subjects and seven patients with liver disease, no choline was demonstrated in 24-hour specimens of urine. In four normal subjects, 5.70 to 11.00 mg. of TTMA N and in 22 patients with liver disease, 5.80 to 14.99 mg. of TTMA N were found in 24-hour specimens of urine.

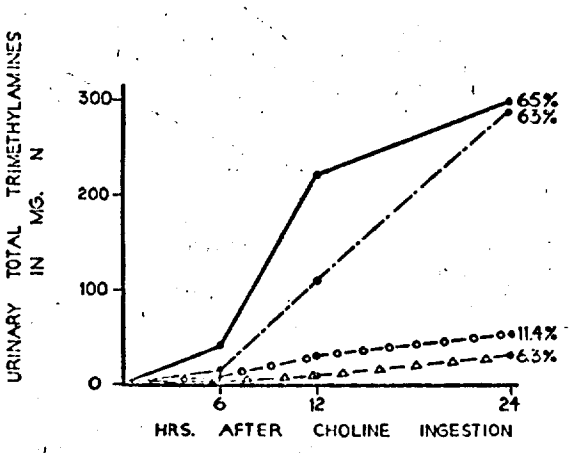

FIG. 3. URINARY EXCRETION OF TRIMETHYLAMINES AFTER ORAL ADMINISTRATION OF 4 GRAMS OF CHOLINE BASE TO A NORMAL PERSON WITHOUT SUPPLEMENT (————), AFTER FOUR DAYS TREATMENT WITH AUREOMYCIN AND SULFATHALIDINE (—○—○—), WITH SIMULTANEOUS ADMINISTRATION OF 100 GRAMS CANE SUGAR (—·—·—) AND 100 GRAMS OF STARCH (—△—△—)

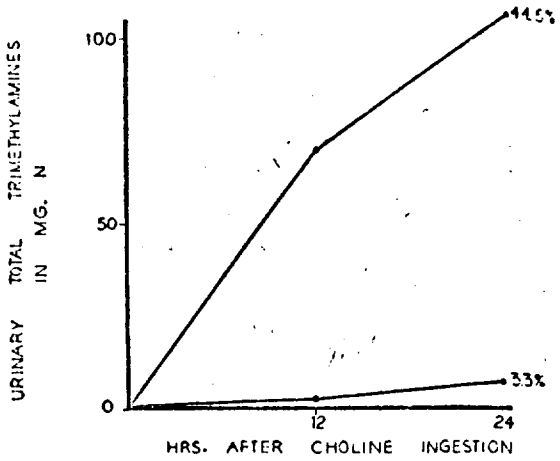

FIG. 4. URINARY EXCRETION OF TRIMETHYLAMINES BY A PATIENT WITH HEPATIC CIRRHOSIS AFTER ORAL ADMINISTRATION OF 2 GRAMS CHOLINE BASE BEFORE (UPPER LINE) AND AFTER (LOWER LINE) FOUR DAYS TREATMENT WITH AUREOMYCIN AND SULFATHALIDINE

Response to administration of choline: After administration of 8 grams of choline base to one normal individual, equal quantities of choline were excreted in any period observed. During the first 24 hours the total excretion was 1.40 mg. choline

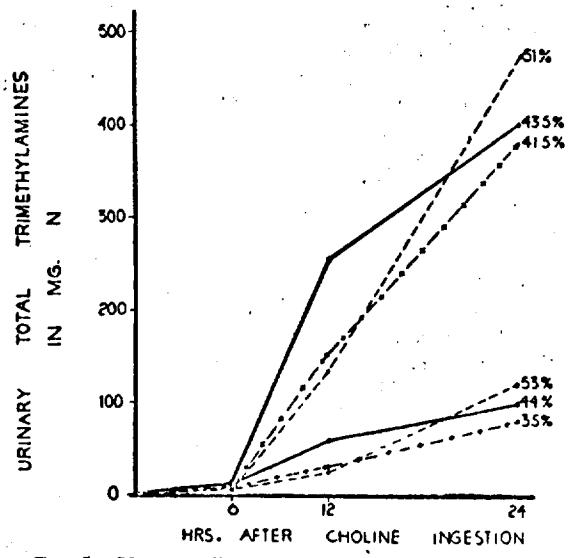

FIG. 5. URINARY EXCRETION OF TRIMETHYLAMINES BY THREE PATIENTS WITH LIVER DISEASE AFTER ADMINISTRATION OF 8 GRAMS (UPPER LINE) AND 2 GRAMS (LOWER LINE) CHOLINE BASE

Each type of line refers to the same patient.

N. Upon administration of 2 or 4 grams of choline base to four normal individuals, 0.75 to 1.60 mg. choline N were excreted in the urine. After administration of 2 to 8 grams of choline base to four normal subjects (in six experiments [Figure 2]), 60 to 67 per cent of choline N was excreted within 24 hours as TTMA of which 95 to 97 per cent was TMAO. This percentage was independent of the amount administered. Relatively little was excreted before six hours and after 12 hours, the bulk being excreted between six and 12 hours (Table II).

Effect of ingestion of other methyl compounds: Administration of 4 and 8 grams of betaine chloride or of 20 grams of dl-methionine to a normal individual did not cause urinary excretion of either choline or betaine and did not increase TTMA excretion.

Influence of intestinal bacteria upon the excretion of TTMA: Since previous investigations (10) indicated that choline is metabolized to TMA by species of the genera *Proteus, Salmonella, Eberthella* and *Shigella,* the TMA formation was determined by the aeration method in tryptose media which contained 1 per cent of choline chloride and had been inoculated with 1 drop of 1:1,000 saline dilution of feces. In five samples, 41 to 60 per cent of the choline present was converted to TMA in 24 hours. If 0.3 gram or less choline chloride was present in 100 ml. of tryptose medium, all choline was converted to TMA. The urinary excretion of TTMA was determined in one normal person and in two patients with cirrhosis of the liver before and after administration of 0.5 gram of aureomycin and 1 gram of sulfathalidine every four hours for four days including the day of the second choline administration. In all three instances the excretion of TTMA was greatly reduced (Figures 3 and 4 representing two of the instances). In the third one (a patient with cirrhosis) only 12-hour urine collections were available and 17.1 mg. of TTMA N instead of 78.5 mg. (without antimicrobial treatment) were excreted. Feces of the experimental subjects after treatment with aureomycin and sulfathalidine proved to be almost free of bacteria.

TABLE III

Urinary excretion of choline and trimethylamines in patients with hepatobiliary diseases within 24 hours after administration of 2 grams of choline base

Diagnosis	Choline		Total trimethylamines			
	Mg. N	Per cent of choline N fed	Mg. N	Per cent of choline N fed	Per cent excreted in first 12 hours	(Choline/TMA) ×100
Obstruct. jaundice	1.40	0.65	152.0	66.0	46.0	0.92
Obstruct. jaundice	1.30	0.56	77.1	33.6	41.5	1.70
Obstruct. jaundice	0.79	0.34	140.0	60.5	67.0	0.56
Infect. hepatitis	2.38	1.04	61.0	26.5	36.0	3.80
Infect. hepatitis	1.18	0.51	106.0	46.0	55.0	1.12
Infect. hepatitis	0.75	0.35	148.1	64.0	31.0	0.51
Infect. hepatitis	0.62	0.27	101.4	44.2	9.2	0.61
Infect. hepatitis	1.90	0.83	91.7	39.8	13.2	2.08
Chronic hepatitis	0.31	0.13	144.1	63.0	24.7	0.22
Chronic hepatitis	0.59	0.26	122.4	53.0	20.3	0.48
Cirrhosis	—	—	106.8	45.5	70.0	—
Cirrhosis	0.70	0.30	87.9	38.2	33.1	0.79
Cirrhosis	0.77	0.34	80.2	35.0	34.2	0.96
Cirrhosis with jaundice	1.10	0.48	28.6	12.4	42.0	3.85
Cirrhosis with jaundice	1.02	0.44	25.0	10.9	72.0	4.00
Cirrhosis with jaundice	—	—	80.5	35.0	38.0	—
Cirrhosis with ascites	0.65	0.28	101.0	44.0	29.5	0.64
Cirrhosis with jaundice and ascites	1.35	0.59	82.5	35.8	61.0	1.64
Cirrhosis with jaundice and ascites	1.15	0.65	26.2	11.4	61.0	4.40
Cirrhosis with jaundice and ascites	—	—	25.6	11.2	39.0	—
Cirrhosis with jaundice and ascites	1.02	0.44	82.1	35.7	39.4	1.22
Cirrhosis with jaundice and ascites	0.85	0.37	49.5	21.5	18.2	1.73

It has been shown that glucose inhibits the conversion of choline to TMA by bacteria (10). Tryptose-choline media to which 1 per cent of glucose was added, failed to reveal upon inoculation with fecal material, any TMA formation. One normal subject who received simultaneously with 4 grams of choline base, 100 grams of cane sugar, excreted in the first 12 hours half of the TMA excreted previously without sugar administration; after 24 hours the excretion did not differ from the previous test (Figure 3).

Administration of 100 grams of starch simultaneously with choline decreased the TTMA excretion in one normal person (after ingestion of 4 grams of choline base) to one-tenth, even after 24 hours (Figure 3). In a second instance (which is not graphically presented), the TTMA excretion after administration of 2 grams of choline base decreased following starch intake from 61 to 17.4 per cent of the choline N given. The urinary choline excretion did not increase.

Excretion of choline and TTMA in patients with liver diseases

In patients with liver diseases the urinary excretion of choline after administration of 2 grams of choline base was not higher than in normals. In the three patients who received 8 grams of choline base, the amount of choline excreted was higher (between 2.6 and 3.4 mg. choline N) but did not exceed one-third of 1 per cent of the total amount given.

After administration of 2 grams of choline base, two out of three patients with obstructive jaundice had a normal excretion of TTMA while the third, who died a few days later, excreted considerably less. Of seven patients with infectious hepatitis, five excreted considerably less TTMA than normal. In all 10 cases of cirrhosis investigated (two studied twice), the excretion was markedly decreased; in the two instances studied on two occasions, almost identical results were obtained. Moreover, in almost all patients with hepatic disease, the excretion was delayed in contrast to the normals. In the latter, the bulk of TTMA was excreted in the first 12 hours after the intake of choline while in the patients it was excreted between 12 and 24 hours. This delay was apparent even if the total amount excreted per 24 hours was not significantly decreased (Table III).

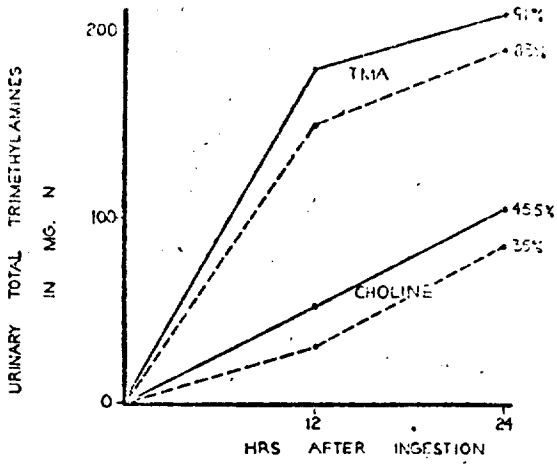

FIG. 6. URINARY EXCRETION OF TRIMETHYLAMINES AFTER ADMINISTRATION OF CHOLINE BICARBONATE AND TRIMETHYLAMINE HYDROCHLORIDE (BOTH IN AMOUNTS OF 230 MG. N) BY TWO PATIENTS WITH HEPATIC CIRRHOSIS

Each type of line refers to the same patient.

In three patients with liver disease, the percentage of TTMA N excreted in relation to the amount of choline N ingested was not significantly changed when 8 grams instead of 2 grams of choline base were given (Figure 5). Two patients with liver disease who previously had delayed and reduced TTMA excretion after choline administration and who ingested TMA hydrochloride in amounts equivalent to 230 mg. N excreted it rapidly and almost completely (Figure 6).

DISCUSSION

In contradiction to the previously cited report on urinary choline excretion (1), this substance is excreted in the urine in insignificant amounts by normal subjects and patients with liver diseases with or without administration of large doses of choline. With the method used, the excretion rarely exceeded one-third of 1 per cent of the administered dose. Moreover, reasonable doubt exists as to whether the very small precipitate represents choline and the figures given are maximum rather than actual amounts. The bulk of the reineckate obtained in the urine after choline administration (independent of the quantity administered) was proven to be trimethylamine (TMA). The greatest part of TMA was in the oxide form. This is in keeping with previous reports that in

rats (11) and in one human (12) after choline administration, TMA and TMAO appeared in the urine.

This study indicates that, as measured by two different methods, two-thirds of the choline administered appears in the urine as TMA or its oxide. The absolute amount is astonishingly well duplicated in the same person. Since many bacteria, especially those of the *Proteus* and *Shigella* groups, are known to transform choline into TMA (10), the possibility of such a process in the intestinal tract had to be considered. It was confirmed by the observation that *in vitro* feces transform choline, to a large degree, to TMA and that suppression of the intestinal flora by antimicrobial agents greatly inhibits the urinary total trimethylamines (TTMA) excretion without influence upon the choline excretion. The conclusion is justified that two-thirds of choline administered as dietary supplement, independent of the dose given, is transformed in the intestinal tract into TMA. Information as to the lipotropic activity of the latter is not available. Neither betaine nor methionine is transformed or excreted as TTMA. The fate of the remaining one-third of choline in the body is unknown.

The concept of an extensive bacterial transformation of choline into TMA appears strange in view of the fact that choline absorption should take place in the upper part of the small intestine while significant bacterial activity starts in the large intestine. Further investigations should clarify the extent of absorption of choline as such in different species.

Glucose, which is known to suppress TMA production from choline by bacteria, inhibits *in vitro* production of TMA by feces. Therefore, the suppression of intestinal TMA formation (as measured by urinary TTMA excretion) by administration of choline together with carbohydrates was attempted. Cane sugar, which is rapidly absorbed from the intestine, delayed the TTMA excretion (and probably the bacterial transformation) without reducing the total excretion in 24 hours. In contrast, starch given with choline for the entire 24-hour period considerably depressed the bacterial transformation of choline if the urinary excretion of TTMA is a measurement.

Three possible explanations have to be considered for the observed reduction or delay of the urinary TTMA excretion after choline intake found in hepatic diseases:

(*a*) Impaired absorption of TMA in the presence of liver damage. The almost complete urinary excretion of ingested TMA even in patients with liver disease speaks against this assumption.

(*b*) Faulty urinary excretion of TTMA. The complete excretion of fed TMA in liver diseases contradicts this possibility also. The bulk of TMA is excreted as its oxide. The possibility, therefore, has to be considered that the oxidized form is more readily eliminated by the kidneys; if the liver performs this oxidation, in liver diseases this function could be at fault. *In vitro* studies (13) have indicated that liver tissue reduces TMAO to TMA; therefore, the fact that the ratio between urinary TMA and TMAO is not significantly altered in liver diseases contradicts this assumption.

(*c*) Faulty bacterial action in liver diseases. Possibly the bacterial flora of the intestine is altered in patients with hepatobiliary diseases in a quantitative and/or qualitative manner which might explain the reduced formation of TMA in these conditions.

The data so far available are too scant to permit conclusions about the therapeutic value of supplementing choline therapy with antimicrobial agents or carbohydrates or about the causes of choline deficiency in the human. The utilization of orally administered choline as measured by phospholipid turnover with P^{32} (14) cannot, at present, be correlated with the urinary excretion of TTMA since the latter does not reflect the fate of absorbed choline in the body. For this purpose, studies on the urinary excretion of intravenously administered choline, which are at present under way, appear more promising.

SUMMARY

1. A procedure was adopted for the determination of choline in the urine using its precipitation by Reinecke salt, and procedures were developed for the determination of trimethylamine and trimethylamine oxide in the urine, based on either Reinecke salt precipitation or on the Folin-Ciocalteu reaction. These procedures were applied to the urines of four normal persons and 22 patients with hepatobiliary diseases before and after oral administration of 2 to 8 grams of choline base.

2. Choline was not found in the urines of normal persons nor patients with hepatobiliary diseases under basal conditions. After oral administration of choline, very small amounts, if any, of choline were excreted, as a rule not exceeding 0.3 per cent of the amount of choline administered.

3. Under basal conditions, small amounts of trimethylamines were found in the urine. Within 24 hours about two-thirds of the choline N ingested was excreted in normal individuals as trimethylamine and its oxide.

4. Incubated stool dilutions transformed choline to trimethylamine. Inhibition of the intestinal flora by aureomycin and sulfathalidine greatly depressed the urinary trimethylamine excretion. This suggests that the urinary trimethylamines result from bacterial transformation of choline in the intestine. The greater part of therapeutically administered choline is, therefore, changed in the liver into a product without known lipotropic activity.

5. Administration of cane sugar with choline delayed urinary trimethylamine excretion, while intake of starch depressed it.

6. In liver diseases the urinary trimethylamine elimination after choline administration is delayed or decreased. Ingested trimethylamine in these patients is readily excreted.

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Action of Enterobacteriaceae on Choline and Related Compounds

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ABSTRACT

The ability of certain of the Enterobacteriaceae to produce trimethylamine from choline has been demonstrated. This provides an additional biochemical test for bacterial taxonomy.

In recent years choline has become recognized as an essential dietary component and its role in the nutrition and metabolism of various animal species has been intensively studied (Best and Huntsman 1932, Kaplan and Chaikoff 1937, Griffith and Wade 1939, Chandler and du Vigneaud 1940). Frequent reference is made to the bacterial degradation of this compound (Waksman 1932, Buchanan and Fulmer 1930, Plimmer 1933, Gortner 1938) despite the fact that few carefully controlled investigations with pure cultures in well defined media have been carried out. Gulewitsch (1898) suggested without experimental evidence that microorganisms would decompose choline and neurine. Supplee (1919), Cusick (1920), Sommer and Smit (1923) found that *Bacillus ichthyosmius* produced trimethylamine from the choline in lecithin. Poller (1933) using a mixed culture obtained from decomposing pancreas, reported trimethylamine, ethyl alcohol, and neurine as products of choline decomposition.

The present study is a continuation of other work on the formation of trimethylamine from its oxide by members of the Enterobacteriaceae (Wood and Baird 1943; Wood, Baird and Keeping 1943). A part of the findings to be reported here have already been published as a note (Wood and Keeping 1944).

EXPERIMENTAL

Cultures

Most of the cultures were those which had been used in the trimethylamine oxide investigation (Wood and Baird 1943). In addition 126 strains from the colon-aerogenes group were freshly isolated from Halifax harbour water and from cod fillets. The cultures were checked for purity from time to time during the course of the investigation.

Medium

The following medium has been found suitable for most species of the Enterobacteriaceae, although certain of the *Erwinia* did not grow well in it: Bactopeptone 5 g., $MgSO_4.7H_2O$ 1g., NaCl 5 g., K_2HPO_4 1 g., choline HCl 5 g., and

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distilled water 1000 c.c. Choline hydrochloride (Eastman Kodak Co.) was used. The other inorganic constituents were c. p. quality. The pH of the medium was 7.1 before autoclaving. After autoclaving at 11 pounds pressure for 25 minutes the pH was 7.2.

Choline Decomposition

It was found that trimethylamine was produced by the bacterial decomposition of choline. *Shigella alkalescens* grown in the above medium with excess $CaCO_3$ at 37°C. for 20 hours produced 1950 mg. trimethylamine per litre or 90 per cent of the theoretical yield of 2107 mg. Organic acid was also produced from the choline, unbuffered choline broth changing from pH 7.2 to pH 4.5 in seven hours at 37°C. Trimethylamine production was markedly inhibited by the presence of an available carbohydrate in the medium. Glucose, 0.1 per cent, inhibited the trimethylamine production 50 per cent, and with 0.2 per cent glucose only 25 per cent of the trimethylamine formed in the base medium was obtained. These findings suggest that in the bacterial metabolism of choline the alcoholic side chain is oxidized as a source of energy and the trimethylamine set free as a by-product of the splitting of the molecule. This is in contrast to the role of choline as donor of methyl groups in animal metabolism (du Vigneaud, *et al.* 1941).

Trimethylamine was determined colorimetrically (Dyer 1945) and by vacuum distillation (Beatty and Gibbons 1937). Trimethylamine was also isolated as the hydrochloride from *Shigella alkalescens* cultures in the basal medium. Routine determinations were made by the qualitative trimethylamine method of Wood and Baird (1943). Cultures were always grown in the base medium devoid of choline as negative controls, and choline broth cultures of *Shigella alkalescens* were used as positive controls. At no time was a positive test obtained in the absence of choline in the broth.

Growth Conditions

The various species were grown at their optimum temperature until adequate growth was obtained as judged by turbidity. It was usually found that trimethylamine could readily be detected by the qualitative test after 24 hours incubation. Certain species such as *Shigella alkalescens* gave positive tests after only 12 hours growth.

RESULTS

From one to six strains of the following *Salmonella* were tested: *S. choleraesuis*, *S. schottmuelleri*, *S. typhimurium*, *S. abortivoequina*, *S. abortus ovis*, *S. enteriditis*, *S. paratyphi*, *S. paratyphosa* C, *S. pullorum*, *S. hirschfeldii*, *S. paratyphi* A., *S. anatis*, *S. typhisuis* (Poppe), *S.* sp. (Kentucky), *S.* sp. (Newport), *S.* sp. (London), *S.* sp. (Poona), *S.* sp. (Minnesota) *S.* sp. (Bredeney), *S.* sp. (*Vi typhosa*), *S.* sp. (Ballerup), *S.* sp. (Hvittingfoss), *S.* sp. (Onderstepoort), *S.* sp. (Cerre), *S.* sp. (Rubislaw), *S.* sp. (Tel-avi), *S.* sp. (Kerkee), *S.* sp. (Carrau), *S.* sp. (Worthington), *S.* sp. (Urbana), *S.* sp. (Senftenberg), *S.* sp. (Derby), *S.* sp. (Reading), *S.* sp. (Thompson), *S. aertrycke*, *S. paratyphi* B. All were negative.

The results obtained with other members of the family Enterobacteriaceae are shown in the table below. "Positive" denotes trimethylamine production from choline. The generic and specific names used in the table are those which the various cultures carried when received at this laboratory.

Species tested	Cultures	
	No. positive	No. negative
Escherichia coli	10	135
" *freundii*	1	12
Aerobacter aerogenes	40	3
Klebsiella ozaenae	0	1
Erwinia amylovora	1	21
Serratia marcescens	0	20
" *indica*	0	2
" *plymouthensis*	1	0
Proteus rettgeri	51	0
" *morganii*	0	9
" *ichthyosmius*	5	0
" *vulgaris*	13	0
" *mirabilis*	12	0
" *hydrophilus*	0	5
Eberthella typhosa	0	13
Shigella alkalescens	84	0
" *dysenteriae*	0	12
" *paradysenteriae*	0	87
" *paradysenteriae* (Boyd)	0	2
" *ambigua*	0	9
" *schmitzii*	0	1
" sp. (Newcastle)	0	5
" *equirulis*	0	1
" *sonnei*	0	27
" *madampensis* (dispar)	0	21
" *ceylonensis*	0	2

DISCUSSION

The genera *Serratia*, *Salmonella*, *Eberthella* and *Erwinia*, with two exceptions, failed to produce trimethylamine from choline. This is in contrast to their ability to produce this amine from trimethylamine oxide (Wood and Baird 1943).

Ninety-three per cent of the *Aerobacter aerogenes* cultures decomposed choline while only six per cent of the *Escherichia coli* strains did so. The division of these two species by means of the choline test appears to be quite sharp. Further work with a greater number of strains of each species might reveal the proper position of the exceptions in this group. Stuart, *et al.* (1943) obtained an almost perfect biochemically intergrading series of cultures from *Shigella alkalescens* to *Escherichia coli* illustrating what appeared to be an evolutionary trend of *S. alkalescens*. The production of trimethylamine from choline by all *S. alkalescens* strains and some strains of the coliform group is further evidence of a close relationship between *S. alkalescens* and this group.

If the reclassification of *Proteus hydrophilus* as *Pseudomonas punctata* (Guthrie and Hitchner 1943) or as *Aeromona hydrophila* (Stanier 1943) or the removal of *P. hydrophilus* and *P. ichthyosmius* from the genus *Proteus* (Borman, Stuart and Wheeler 1944) is granted, then *Proteus morganii* is the only member of the genus *Proteus* which produces trimethylamine from choline.

In the genus *Erwinia* a single positive culture was obtained out of 22 tested. The members of this genus grew poorly in the test medium and for this reason it is suggested that further work should be undertaken before definite conclusions are drawn.

Betaine and acetyl choline bromide were tested with choline positive cultures. In no case were any of the species tested able to produce trimethylamine from betaine. The same results were obtained with acetyl choline bromide as with choline. The *Proteus* group grew well in the acetyl choline bromide broth but a number of strains of several *Shigella* species grew scantily or not at all. Further work on the compounds related to choline is necessary.

Attempts were made to "train" negative cultures of *Proteus morganii, Shigella ambigua, S. dysenteriae, S. paradysenteriae, S. sonnei* and *S. gallinarum* by 60 serial transfers in choline broth. In no case did a negative culture become positive under this treatment. Positive cultures carried in broth devoid of choline did not lose their ability to decompose this compound.

The presence of bacteria of this family on fish and fish products is frequently noted. Since certain of these are able to produce trimethylamine from choline it is possible that part of the trimethylamine arising in fish spoilage may come from this source although the carbohydrate present in fish muscle would probably suppress the reaction. Choline occurs in cod muscle to the extent of 78 mg. per 100 g. (Fletcher, Best and Solandt 1935). Choline is thus a potential source of approximately 9 mg. trimethylamine nitrogen per 100 g. cod muscle. Since fourteen times as much trimethylamine nitrogen may be produced from the trimethylamine oxide present (Beatty 1939) and since a larger number of the contaminants usually present on fish reduce trimethylamine oxide (Wood and Baird 1943; Baird and Wood 1944) than decompose choline, trimethylamine production from fish muscle choline is probably of little practical significance.

The demonstration that certain of the intestinal bacteria are capable of decomposing choline would suggest that close attention be paid to the possible interference of bacteria in nutritional experiments with choline.

SUMMARY

Certain members of the family Enterobacteriaceae produced trimethylamine from choline. All strains of *Proteus rettgeri, P. ichthyosmius, P. vulgaris, P. mirabilis* and *Shigella alkalescens* were positive. All strains of *P. morganii, P. hydrophilus, Salmonella, Eberthella*, and all *Shigella* except *S. alkalescens* were negative. Exceptions were encountered in *Escherichia, Aerobacter, Serratia* and *Erwinia*.

No trimethylamine was obtained from betaine.

The same results were obtained with acetyl choline bromide as with choline.

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Effect of Choline Chloride on Development of Atherosclerosis in the Rabbit.* (18033)

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The demonstration of the lipotropic activity of choline has led to the testing in experimental animals of its ability to prevent the arterial lipid deposition characteristic of atherosclerosis. Although some observers(1,2) have failed to find any protective action, others(3-6) have claimed that choline administration retards the development of lesions.

In investigating this subject we have administered choline chloride to rabbits by force-feeding gelatin capsules rather than by mixing choline with the food, not only to ensure a known and uniform dosage, but also in order to avoid a possible depression in the food intake of choline-treated animals due to the brackish taste of the chloride. It has previously been shown(7) that caloric restriction with consequent weight loss or decreased rate of weight gain inhibits the development of atherosclerotic lesions.

Methods. Fourteen adult male rabbits of various breeds, kept in individual cages and allowed Purina Rabbit Chow Checkers and water *ad libitum*, were used as experimental

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TABLE I.
Summary of Experimental Data.

Animal	Avg blood total cholesterol (mg %)	Initial wt (kg)	Ratio of final to initial wt	Avg food consumption per kg body wt (g per day)	Degree of atherosclerosis
1	101	3.79	1.12	41	0
2*	109	3.28	1.15	37	0
3	117	3.29	1.25	46	0
4	124	3.03	.96	35	1
5*	140	3.40	.93	32	1
6*	149	3.25	.96	30	2
7	154	3.32	1.08	40	2
8	166	3.39	.96	32	3
9*	202	3.35	.87	27	3
10	235	2.78	1.03	38	2
11	238	3.56	.92	35	3
12	271	3.26	.91	25	4
13	320	3.72	.81	25	4
14*	329	2.99	.93	31	3
		Means and Standard Errors.			
Choline	185.8 ± 38.8	3.254 ± .071	0.968 ± .048	31.4 ± 1.6	1.80 ± .58
Control	191.8 ± 25.6	3.349 ± .106	1.004 ± .044	35.2 ± 2.4	2.11 ± .51

* Animals given choline.

animals. Each animal received 1 g of cholesterol 6 days a week enclosed in 2 size 00 gelatin capsules. Five animals received, in addition, 1 g of choline chloride 6 days a week in capsules. The 9 controls received 2 empty capsules 6 days a week. In using this method of administration, suggested by Pollak(8) for cholesterol feeding, some difficulty was encountered in getting the animals to swallow the capsules and several rabbits were lost from respiratory obstruction before a satisfactory technic was developed. Good results were obtained when the capsule being administered was gently introduced into the pharynx while the animal's head was displaced (not rotated) backwards. Each animal was weighed weekly, its food consumption recorded daily, and its blood total cholesterol level determined weekly on heparinized ear vein blood by the Schoenheimer-Sperry method(9). After 9 weeks of cholesterol and choline administration the animals were killed and the whole aortas removed and fixed in 10% formalin. The aortas were stained with Sudan IV and graded as unknowns into 5 arbitrary groups according to the area of intima involved by lesions.

Results and discussion. The data are summarized in Table I in order of increasing average blood cholesterol level. Animals given choline are indicated by asterisks. The blood cholesterol averages do not include determinations made before the beginning of cholesterol administration. Food consumption values are averages of the 9 weekly averages computed for each animal on the basis of its average daily food intake and average body weight each week.

There is an obvious increase in the degree of atherosclerosis with increasing average blood cholesterol level. The two rabbits (10 and 14) whose degrees of atherosclerosis are less than would be expected from consideration of blood cholesterol levels alone had the lowest initial weights of the group. This is consistent with our previous finding(7) that animals of low initial weight tend to develop less atherosclerosis than animals of high initial weight, other factors being equal. However, with the foregoing exceptions, the variations in initial weight, weight change, and food consumption in the present experiment are relatively small and are insufficient to affect appreciably the correlation between degree of disease and blood cholesterol level.

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The means of the average blood cholesterol levels, initial weights, ratios of final to initial weight, food consumptions, and degrees of atherosclerosis in the choline-treated and control groups do not differ significantly.

The statistical significance of the difference between the mean degrees of atherosclerosis in the two groups adjusted to the common mean blood cholesterol level can be tested by the analysis of covariance. The adjusted means do not differ significantly, since the variance ratio F (0.23) is well short of the 5% point (4.84). Moreover, it is unlikely that any feasible increase in the number of experimental animals would lead to the demonstration of a significant difference under these experimental conditions; for it can be estimated that control and test groups of approximately 110 animals *each* would be required to demonstrate significance at the 5% level assuming that the relative standard deviation will be the same for the larger sample(10).

In a recent investigation(6), the results of which indicated that choline retarded the development of lesions, Steiner offered 1 g of cholesterol mixed with the food 3 times weekly to 54 rabbits. Ten were offered 1 g and 19 were offered 0.5 g of choline chloride daily mixed with the food. The remaining 25 served as controls. Assuming that choline was offered 6 days a week, and, assuming that equal proportions of the cholesterol and choline offered were actually ingested, the ratio of the cholesterol and choline weekly dosages in the 19 animals offered 0.5 g of choline daily was $3/3 = 1$. In our experiment, the ratio of cholesterol and choline weekly dosages was $6/6 = 1$, so that we believe our choline dosage relative to cholesterol dosage was comparable to that used by Steiner in his animals on the lower choline dosage.

A larger dosage of choline than we have used may inhibit the atherosclerotic process. However, in view of earlier findings(7), we believe that, in order to demonstrate that any agent specifically inhibits the development of lesions independently of any effect on blood cholesterol level or body weight, it should be shown that the treated and control groups do not differ significantly in duration or level of cholesterol administration, in average blood cholesterol level, in initial weight, or in relative weight change during the experiment.

Summary. The degree of aortic atherosclerosis found in 14 male rabbits given 1 g of cholesterol in gelatin capsules 6 days a week for 9 weeks showed a highly significant positive linear correlation with the average blood total cholesterol level.

Five of these animals which received 1 g of choline chloride in gelatin capsules 6 days a week for the 9 weeks did not differ significantly from the 9 control animals in average blood cholesterol level, in body weight, or in the degree of atherosclerosis which developed.

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The Chick's Response to Choline and its Application to an Assay for Choline in Feedstuffs

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DURING our studies on choline assay methods a chick bioassay was needed to determine which of several analytical methods gave results which more nearly paralleled the biological activity. The young chick requires about 1300 mg. choline per kilogram of diet when the ration contains 2750 Calories metabolizable energy per kilogram (Bird *et al.*, 1966). Combs (1963) has related choline requirements to the fat content of the diet.

High energy broiler diets usually contain more than the above NRC requirement figure, and practical experience indicates that somewhat more choline is needed (Fritz, 1965a). Classen *et al.* (1965) concluded that typical broiler diets needed at least 1650 mg. choline chloride per kg. for best performance. As the energy content of the feed is increased, several factors increase the need for dietary choline: the higher energy ingredients contain less choline, the chick consumes less feed per unit gain in weight, and the increased fat content may increase the need for dietary choline.

TABLE 1. *Composition of basal diet*

Ingredients	%
Glucose monohydrate[1]	46.85
Corn gluten meal (42% protein)	15.00
Dried skimmilk	10.00
Dehulled soybean meal	5.00
Casein[2]	5.00
Gelatin[3]	5.00
Blood meal	5.00
Corn oil[4]	5.00
Dicalcium phosphate	2.50
Iodized salt	0.50
Vitamin premix[5]	0.10
Trace mineral premix[6]	0.05

[1] Cerelose—Corn Products Co., Philadelphia, Pa.
[2] Vitamin Test Casein—General Biochemicals, Chagrin Falls, Ohio.
[3] U-COP-Co brand—Wilson & Co., Chicago, Ill.
[4] Mazola—Corn Products Co., Philadelphia, Pa.
[5] The vitamin premix was prepared from crystalline or highly purified components, to furnish per kg. of diet: 5,000 I.U. vitamin A, 1,500 I.C.U. vitamin D_3, 5 I.U. vitamin E, 0.4 mg. menadione, 3 mg. thiamine, 3 mg. riboflavin, 5 mg. pantothenic acid, 30 mg. niacin, 3 mg. pyridoxine, 0.1 mg. folic acid, 0.02 mg. vitamin B_{12}, and 5 mg. procaine penicillin.
[6] Dawes Trace Mineral Compound, which furnished per kg. diet: 64 mg. manganese, 50 mg. zinc, 25 mg. iron, 4 mg. copper, 1 mg. iodine, and 0.25 mg. cobalt.—Dawes Laboratories, Chicago, Ill.

The purposes of this study were to compare the biological response with the results of chemical and microbiological assays for choline, to investigate the chick's quantitative requirements for choline, and to observe the symptoms of choline deficiency in young chicks.

MATERIALS AND METHODS

The studies were made with duplicate groups of day-old straight run White Rock chicks, unless otherwise noted. Ten to thirteen chicks were used per group. They were raised in wire floored, electrically heated starting batteries. The composition of the basal diet is shown in Table 1. From the data of Titus (1961) this diet was calculated to contain 3483 Calories metabolizable energy per kilogram and was believed to meet the known nutritional requirements of the chick except for choline. Methionine and cystine were kept marginal because of the interrelationship between choline and methionine (Quillin *et al.*, 1961), although Henderson and Henderson (1966) concluded that chicks do not transfer methyl groups from methionine to choline. Assays by the microbiological method of Horowitz and Beadle (1943) indicated that this basal diet contained 465 mg. choline chloride per kilogram.

The test diet and tap water were kept before the chicks at all times. Four feeding tests were made.

In the first trial 3 diets were fed to the chicks during a 4 week test period: (1) basal diet, (2) basal diet plus 500 mg. choline chloride per kg., and (3) basal diet plus 1000 mg. choline chloride per kg. The chicks were weighed by groups at weekly intervals. At the end of the test period they were graded for incidence and severity of perosis, a symptom of choline deficiency (Jukes, 1940). A value of zero was assigned for normal legs, and higher numbers indicated increasing damage to the hock joints and to the long bones of the legs. A value of 4 was assigned to those chicks in which the deformity was so great that they could move about only on their hock joints. The chicks were then killed with chloroform and the livers were removed. Group composite samples of the livers were analyzed for fat content by the method of Folch *et al.* (1957).

The second feeding trial was similar except that it lasted 3 weeks and 9 levels of supplemental choline, from 0 to 1200 mg choline chloride per kg., were fed. Five feed samples were also included for comparison to the known quantities of choline chloride. These samples were added at the expense of the basal diet or of ingredients to minimize the effect upon protein and energy content of the diet. At the end of the feeding test the chicks were weighed individually and scored for incidence and severity of perosis. Group data were obtained on feed

TABLE 2.—*Chick performance at three levels of choline*

Dietary choline chloride	465 mg./kg.		965 mg./kg.		1,465 mg./kg.	
Chick group nos.	1	2	3	4	5	6
Av. wt. (g.) Initial	33	33	34	34	34	33
1 wk.	67	68	82	84	98	91
2 wks.	101	104	152	156	198	182
3 wks.	140	157	247	256	308	287
4 wks.	213	229	380	389	459	429
Grams feed/gram gain	2.34	2.41	1.82	1.82	1.79	1.66
Av. perosis score	3.0	3.0	1.9	2.0	0.5	0.4
Liver fat, % fresh wt.	2.95	3.23	3.41	3.48	3.20	3.41
Mortality, %	20	0	10	10	0	0

consumption and compared with the weight gains. Representative chicks from some groups were examined for liver fat (Folch *et al.*, 1957), blood cholesterol (Abell *et al.*, 1952), hematocrit value by the capillary tube centrifuge method, and prothrombin time (Woody *et al.*, 1963). Toe ash content (Baird and MacMillan, 1942) was also determined to assure that the leg weakness was due to perosis and not to suboptimal calcification.

Two additional feeding trials were made to verify the initial observations on (1) liver fat on low choline diets and (2) levels of choline needed for optimum response of chicks by different criteria.

In the third trial 4 groups of chicks were fed diets low in both protein and choline. The protein was reduced to 16.6% by replacing part of the protein concentrates with ground yellow corn. Calculated energy content remained near that of the original basal diet (3467 Calories M.E./kg.). This modified diet was found, by analysis, to contain 266 mg. choline chloride per kg. The 4 test diets were made up with 0, 200, 400, and 600 mg. choline chloride, resp., added per kilogram of feed. After 3 weeks on these diets the chicks were killed and liver fat was determined on group composite samples.

A fourth chick feeding test was made, similar to the second trial described above, but with the dietary choline levels concentrated around the points where maximum growth and minimum incidence of perosis were indicated by the earlier tests. The specific objective was to define more exactly the chick's quantitative requirement for choline. Fourteen levels of supplemental choline chloride, from 900 to 1550 mg. per kg., in 50 mg. increments, were added to the basal diet (465 mg./kg.). After a 3 week feeding period the chicks were weighed individually, scored for incidence and severity of perosis, and liver fat was determined on several group composite samples.

RESULTS AND DISCUSSION

Observations made during the first trial are summarized in Table 2. These results show the chick's response to supplemental choline, demonstrated by weight gains, perosis score, and feed conversion. The effect on growth reached a maximum by 3 weeks of age or less, and indicated that a 3 week feeding test was adequate for further studies.

The second trial was set up to study a wider range of dietary choline levels, to determine optimum levels for various criteria, and to determine the biological response to feed ingredients. Results for the known levels of choline chloride are summarized in Tables 3 and 4.

These observations indicated a graded response in growth, perosis score, and feed

TABLE 3.—*Influence of dietary choline levels on growth, incidence of perosis, and feed conversion*

Mg. choline chloride per kg. diet	Av. chick wt. (g.) at 3 wks.*	Av. perosis score*	Grams feed/ gram gain
462	152±37	2.6±.58	2.65
615	182±47	2.3±.59	2.42
765	214±54	2.4±.86	2.31
915	248±43	2.3±.71	2.05
1,065	272±49	2.0±.82	1.90
1,215	283±66	1.9±.79	1.88
1,365	299±43	1.8±.52	2.01
1,515	305±43	1.1±.72	1.86
1,665	295±45	0.6±.66	1.93

* Arithmetic mean and its standard deviation.

conversion. No marked or consistent effect on liver fat, blood cholesterol, hematocrit, or prothrombin time resulted from variation in the dietary level of choline within the range studied. The toe ash data show that the bones were well calcified.

The livers of all chicks in the first 2 trials appeared normal on macroscopic examination, and the fat content found by analysis was within the normal range. Fatty livers have been considered a general symptom of choline deficiency in various species (Best and Huntsman, 1932; Handler and Dubin, 1946; Johnson and James, 1948; Hove *et al.*, 1954) and have been listed as a symptom of choline deficiency in the chick (Titus, 1961). In many reports in the literature the choline deficiency was complicated by protein deficiency or other factors which contributed to fatty infiltration of the liver.

TABLE 4.—*Miscellaneous observations on chicks which received graded levels of dietary choline to 3 wks. of age**

Mg. choline chloride per kg. diet	Liver fat, % fresh wt.	Cholesterol, mg./100 ml. plasma	Hematocrit, % packed cells	Prothrombin time, seconds	% Ash in dried toes
465	4.1	171	36	18.8	14.87
765	4.2	64	36	20.9	13.99
1,065	2.8	174	33	19.2	13.70
1,365	3.8	137	32	18.6	14.02
1,665	3.2	143	32	19.0	14.30

* Values obtained on group composite samples.

A low protein diet was fed to determine if this would alter the fat content of the liver, or make the maintenance of normal liver fat more dependent upon dietary choline. The 16.6% protein diet did not support maximum growth. Liver fat was slightly higher than that observed on the higher protein diet, but still within normal range and not consistently influenced by the level of dietary choline. With the 266 mg. choline chloride per kg. of feed, the group composite sample of fresh liver was found to contain 5.1% fat. With 200, 400 and 600 mg. added choline chloride the liver fat values were, resp., 5.9, 4.9, and 5.2%.

Under the conditions of these tests, fatty livers were not a symptom of choline deficiency in the chick.

There was a response in these tests in perosis score and in feed conversion, but the effect upon growth was larger and had a lower coefficient of variation. Figure 1 shows the weight of the chicks at 3 weeks of age plotted against the known levels of

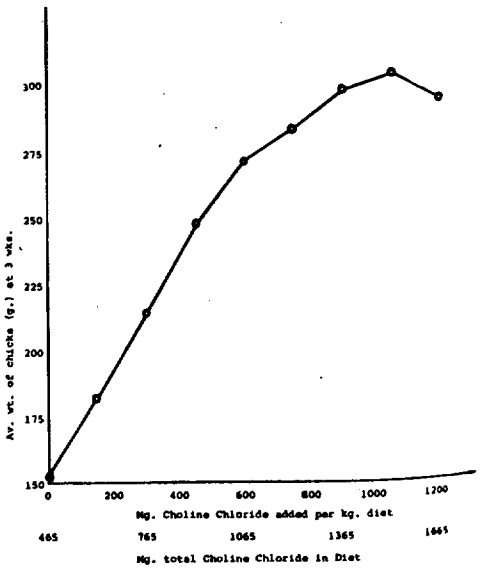

FIG. 1. Chick weight response to supplemental choline.

TABLE 5.—*Response of chicks to feed samples*

Supplement		Replaced in basal diet	Av. chick wt. (g.) at 3 wks.*	Av. perosis score*	Grams feed/ gram gain	Mg. choline chloride per g. sample
Level added	Sample					
15%	Chick starter	15% Basal	190±25	2.5±.50	2.47	2.07
30%	Chick starter	30% Basal	273±40	2.2±.59	1.87	
20%	Degerm. corn meal	16% Cerelose + 4% c. gluten	176±54	2.3±.57	2.40	0.55
50%	Degerm. corn meal	40% Cerelose + 10% c. gluten	188±50	2.2±.43	2.22	
10%	Dehulled soybean meal	10% c. gluten	197±55	2.3±.65	2.43	2.93
20%	Dehulled soybean meal	15% c. gluten + 5% Gelatin	276±58	1.7±.84	1.93	
5%	Dried brewers yeast	5% c. gluten	214±37	2.4±.59	2.14	
10%	Dried brewers yeast	10% c. gluten	236±46	2.1±.53	2.05	4.69
15%	Dried brewers yeast	15% c. gluten	250±46	1.7±.76	2.13	
10%	Dried torula yeast	10% c. gluten	268±48	1.7±.62	1.97	5.98

* Arithmetic mean and its standard deviation.

choline. This reference curve was used to interpret response to the feed samples, and the results of this bioassay are shown in Table 5. Adjustments were made for estimated choline content of ingredients displaced by the sample. Observations on perosis score and feed conversion supported the results shown by growth of the chicks.

Table 6 shows comparison of the chick bioassay results with those obtained by chemical and microbiological methods. The microbiological method of Horowitz and Beadle (1943) gave higher results, but the rapid reineckate method (Fritz, 1965b) gave lower results. The short methanol extraction of the reineckate method may fail to break down a protein-phospholipid complex, and a more vigorous extraction and/or hydrolysis may be needed to measure the choline present in dried yeasts. Engel (1942) showed that prolonged methanol extraction followed by hydrolysis with boiling aqueous barium hydroxide did extract choline from dried yeast. The results of the chick assay on the feedstuffs were in good agreement with values obtained by the Engel method and reported by Engel (1943), by Rhian *et al.* (1943), and by Almquist and Maurer (1951).

A specific dietary level of choline is difficult to select as the chick's quantitative requirement. Considerable interaction takes place between choline and other dietary components (see review by Woods, 1951). For example, betaine is widely distributed in feedstuffs and can serve as a methylating agent (Bird *et al.*, 1966). It must be emphasized that an apparently optimum level of choline applies only to the specific diet used, and caution must be exercised in any attempt to extend the data to other diets and other conditions.

Different criteria indicated different choline requirements for optimum performance. In this test maximum growth was obtained with about 1500 mg. choline chloride per kilogram of diet calculated to contain about 3480 Calories metabolizable en-

TABLE 6.—*Comparison of choline assay methods**

Feed sample	Mg. choline chloride found per gram		
	Chick bioassay	Microbiol. method**	Chemical method†
Chick starter	2.07	2.28	1.66
Degermed corn meal	0.55	0.65	0.48
Dehulled soybean meal	2.93	4.01	2.14
Dried brewers yeast	4.69	4.78	0.38
Dried torula yeast	5.98	7.60	0.60

* In the assays on soybean meal, the results by the chick method were significantly ($P = <.05$) different from those by microbiological or chemical methods. In the assays on the dried yeasts, results by the chick assay or by the microbiological assay did not differ significantly between themselves, but were significantly greater than the results by the chemical assay. Other differences were not statistically significant.

** Neurospora method described by Horowitz and Beadle (1943).

† Reineckate method described by Fritz (1965b).

ergy. Weight differences between the several levels at the top of the growth curve shown in Figure 1 were not statistically significant.

The fourth chick feeding trial was set up to define more precisely the quantitative choline requirement. The results are summarized in Table 7. Growth was a little better than in the earlier trial (Table 3), but the results of the two trials were in good agreement. Again there was a trend toward better growth when the level of choline chloride was 1500 mg./kg. of diet or higher. The incidence and severity of perosis decreased over the entire range of dietary choline used in this test. The last frank cases of perosis (two chicks scored 3 and 2, resp.) were seen on the diet estimated to contain 1865 mg. choline chloride per kg. No chick which received more than this level was scored higher than 1, and the majority were considered normal. Again there was no effect of the choline level on liver fat. A composite sample of livers from chicks on the basal diet (465 mg./kg.) was found to contain 3.7% fat, a sample from an intermediate group (1565 mg./kg.) was found to contain 4.2% fat, and a sample from the highest level of choline used in this test (2015 mg./kg.) was found to contain 4.0% fat.

Optimum growth was obtained with about 1500 mg. choline chloride per kilogram of diet, but at least 1900 mg. were required for essentially complete protection against perosis. A much lower level, i.e., about 1000 mg. choline chloride per kilogram of diet, reduced the feed requirement per unit gain to a minimum.

SUMMARY AND CONCLUSIONS

The chick was found to be an excellent experimental species for choline studies. A graded response, with varying choline intake, was obtained in growth, incidence of perosis, and feed conversion. Choline deficiency had little or no effect on liver fat, blood cholesterol, prothrombin time, hematocrit, or calcification.

Under the conditions of these studies different criteria were satisfied by different

TABLE 7.—*Response of chicks to supplemental choline*

Mg. choline chloride per kg. diet		Av. chick wt. (g.) at 3 wks.*	Perosis score*	Grams feed/ gram gain
Added	Total			
0	465	181±41	2.6±.60	2.36
900	1,365	325±35	1.2±.76	1.72
950	1,415	333±29	1.0±.76	1.69
1,000	1,465	331±42	0.9±.49	1.64
1,050	1,515	344±33	0.6±.39	1.60
1,100	1,565	343±45	0.8±.98	1.72
1,150	1,615	362±53	0.6±.54	1.61
1,200	1,665	347±50	0.5±.59	1.69
1,250	1,715	341±44	0.4±.38	1.66
1,300	1,765	334±35	0.4±.45	1.61
1,350	1,815	356±59	0.5±.57	1.65
1,400	1,865	342±42	0.6±.91	1.65
1,450	1,915	336±34	0.2±.30	1.70
1,500	1,965	332±32	0.2±.37	1.68
1,550	2,015	352±48	0.1±.21	1.76

* Arithmetic mean and its standard deviation.

dietary levels of choline. When choline chloride intake was below about 1000 mg. per kilogram, feed conversion was impaired but no further improvement occurred as the choline content of the diet was increased above this level. The requirement as evidenced by growth was about 1500 mg. per kilogram of cerelose-based diet containing 3480 Calories metabolizable energy. The requirement for protection against perosis was greater, and about 1900 mg. choline chloride per kilogram were required for essentially complete protection.

The chick assay gave choline values which tended to fall between those obtained with the reineckate method and those with the microbiological method, and to agree more closely with the results of the microbiological method when large differences were noted. The chick assay indicated the following choline chloride content, in mg. per gram of sample: chick starter 2.07, degermed yellow corn meal 0.53, dehulled soybean meal 2.93, dried brewers yeast 4.69, and dried torula yeast 5.98.

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A BRONZED PIGMENTATION, ON THE HAIR OF WHITE RATS, INDUCED BY CHOLINE HYDROCHLORIDE

G. M. Higgins, Ph. D., Division of Experimental Medicine, Olive R. Joneson, B. A., Research Assistant, and F. C. Mann, M. D., Division of Experimental Medicine, Mayo Foundation: While studying the character of the anemia induced in rats by choline hydrochloride, we were surprised to observe a bronzing of the hair in a large percentage of our animals. This color change, induced by a reddish brown pigment, apparently adherent to the hair, first appeared around the base of the tail within a few days after administration of choline was begun and gradually extended forward to involve the entire animal after two or three weeks.

In our first experiment, when these changes were observed, the animals were eating a purified diet. The carbohydrate component was sucrose, included at a level of 72 per cent; the protein was vitamin-free casein (Labco brand) at 20 per cent; the fat was corn oil at 4 per cent, and the salt mixture was included at 2 per cent. To provide vitamins A and D, 2 per cent by weight of medicinal cod liver oil was added to the diet. Vitamin B, provided daily by stomach tube to each rat included 100 micrograms of thiamine, 200 micrograms of riboflavin, 100 micrograms of pyridoxine, 200 micrograms of pantothenic acid, 1 mg. of niacin and 10 mg. of choline hydrochloride. The additional amounts of choline* administered were provided in the drinking water at a concentration of 10 mg. per cubic centimeter. Since rats consumed on the average 15 c.c. of water daily, each was taking 150 mg. of choline in addition to the 10 mg. provided in the tubing mixture.

After three weeks, when the rats were well bronzed, two groups of five animals each were selected. To one group of five, brewers' yeast, strain K, was provided in a separate feeding cup, so that rats partook of it when so inclined. Both groups of five continued to eat the high sucrose ration and to take choline in their drinking water. Within a few days the bronzing of the hair began to disappear and in two weeks all rats provided the yeast supplement had normal hair color patterns,

Fig. 1. Bronzed appearance of an animal which had received choline in its drinking water for four weeks. High sucrose diet.

Fig. 2. The same animal two weeks later, having had access to brewers' yeast, strain K. Administration of choline was continued during this two week period. High sucrose diet.

while those not provided the yeast were still extremely bronzed (figs. 1 and 2).

Ten medium-sized male rats, weighing 150 gm., were placed on the purified diet and given choline in their drinking water. Half of these received strain K yeast at a level of 15 per cent in their diet and half of them received the diet without the yeast. Varying degrees of bronzing appeared on all animals which ate the diet without the yeast while those which ate the yeast-supplemented diet did not become bronzed, in spite of the fact that large amounts of choline were taken daily.

*Provided for our use through the courtesy of Dr. A. D. Emmett, Parke, Davis and Company, Detroit, Michigan.

Thirty young male rats, weighing 50 to 60 gm., were arranged into six groups of five rats each. All were provided choline in their drinking water but the diets varied as follows: Group I ate the high sucrose diet, as set forth in the second paragraph. Group II ate a high protein diet in which the casein component was 40 per cent by weight and the sucrose component was 52 per cent by weight. All other constituents were identical with those of the diet of group I. Group III ate the high sucrose diet to which was added 15 per cent by weight of strain K yeast and group IV ate the high protein diet plus the added yeast. Group V ate a diet similar to that of group III except that dried liver at the same level replaced the yeast, while group VI ate the diet of group IV except that dried liver replaced the yeast.

Within three days the rats eating the high protein ration began to bronze and within five days those eating the high sucrose ration began to bronze. Rats of group III, eating the high sucrose diet plus yeast, bronzed very slightly in a week. Those of group IV, eating the high protein diet plus yeast, did not bronze at any time. The intakes of choline were essentially alike, although somewhat greater in groups II and IV than in the other groups. All rats in group V, eating the high sucrose diet plus liver, bronzed extensively. Those in group VI, eating a high protein diet fortified by liver, did not bronze. A protein diet enriched by either yeast or liver appeared to protect all animals against the pronounced bronzing effect induced by choline. Normal increments of body weight were observed in all groups of animals; choline had apparently not modified growth patterns.

Ten young male rats weighing 50 to 60 gm. were selected. All were placed on a high carbohydrate diet in which dextrin replaced sucrose as the source of carbohydrate. Choline was provided in the drinking water as in all tests. Bronzing did not appear in a single animal, even after two weeks. These ten animals were then arranged into two groups of five each. All continued to receive choline but one group of five were fed the high carbohydrate diet containing sucrose and the other group of five continued to eat the diet containing dextrin. In three days all rats eating the sucrose diet began to bronze and in ten days the pattern had extended over the entire body of all five animals, while all dextrin-fed animals remained free of discoloration.

Fifteen young male rats weighing 50 to 60 gm. were selected. Five were placed on a high fat diet, in which the percentage of corn oil was 20, of sucrose 56 and of casein 20. Five were placed on the same high fat diet, except that dextrin replaced the sucrose, and five were placed on the high protein diet in which dextrin replaced the sucrose. All animals received choline in the same concentration in their drinking water. The results were of interest in that all animals eating diets in which dextrin was the source of carbohydrate did not bronze, while the bronzed pattern hitherto described appeared on those eating diets containing sucrose.

Sixteen adult male rats weighing more than 250 gm. were selected. Four were placed on each of the four purified diets: high sucrose, high protein, high sucrose with yeast and high protein with yeast. All were given choline in their drinking water. The daily intakes of choline ranged from 150 to 200 mg. per day. This experiment has continued for four weeks and we have not observed a trace of bronzing in any of these adult rats. It would appear that the adult rat may be protected in some way from the onset of conditions which, in immature rats, so readily induced the pigmentation that we have observed.

Richter and Rice[1] have recently shown that weanling rats grew at normal rates when their only source of the vitamin B complex was the feces collected from normal animals. Accordingly we proposed to feed weanling rats the purified diet, free of vitamin B, and to test the potency of the feces collected from our bronzed animals fed the purified diets and taking choline. Within two or three days, weanling rats eating feces of bronzed animals likewise became bronzed, regardless of whether they were fed the purified high sucrose diet, the high protein diet or the high sucrose diet fortified with yeast. Those eating feces of animals fed the high protein diet fortified with yeast did not become bronzed. Likewise weanlings eating the feces of animals getting choline and fed the high protein diet fortified with liver did not become bronzed. In each instance, bronzing of the weanling rats did not occur when they were fed feces of animals which had not previously become bronzed.

Furthermore, weanling rats were fed feces of rats which had received choline while eating diets in which the source of carbohydrate was dextrin instead of sucrose. These weanling rats did not become bronzed. Likewise, weanling rats fed feces of animals receiving choline while receiving the high fat-dextrin diet did not become bronzed. It will be recalled that these rats from which the feces were derived had not become bronzed as had those receiving choline while eating the high fat-sucrose diet.

These are preliminary observations and an explanation for these findings is not yet available. Since certain diets appear to inhibit the changes that choline induced, we believe that a gastro-intestinal factor may be involved. Studies now projected include an investigation of the nature and source of these pigments, the histopathologic changes in the skin and the influence on the intestinal micro-organisms that may be exerted by the intake of such large daily amounts of choline hydrochloride.

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Acute Toxicity of Choline Hydrochloride Administered Intraperitoneally to Rats.*†

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Choline hydrochloride is not highly toxic. Mott and Halliburton[1] emphasize the low order of toxicity when they state that "We have never succeeded in killing an animal by injection of choline or choline hydrochloride." However, lethal doses for several species have been reported[1-9] as of the order of 40-60 mg per kg body weight for intravenous administration and of the order of 200-1000 mg per kg for subcutaneous administration.

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TABLE I.
Dosage-Mortality Table for Rats Grouped by Dosage Ranges.*
(Intraperitoneal Injection: 518 Rats.)

	Concentration of choline hydrochloride solutions							
	200 mg/ml		100 mg/ml		40 mg/ml		20 mg/ml	
Dosage range mg/100 g body wt	No. rats	% mortality	No. rats	% mortality	No. rats	% mortality	No. rats	% mortality
20-24	15	0						
25-29	44	25						
30-34	31	61	22	32				
35-39	46	72	43	54	8	12		
40-44	15	100	28	79	43	19		
45-49					39	41		
50-54			20	80	11	82	9	67
55-59			5	60			30	43
60-64					15	87	22	64
65-69							6	100
70-74							14	36
75-79							23	71
80-84					15	100		
85-89							14	86
Range of LD_{50}	29-34 mg/100 g body wt		37-38 mg/100 g body wt		41-49 mg/100 g body wt		59-75 mg/100 g body wt	

* The statistical work was performed by Elizabeth Street, her assistance is gratefully acknowledged.

In the first series of tests, 4 solutions of choline hydrochloride (Eastman Kodak Co.) of concentrations 200, 100, 40 and 20 mg per ml, respectively, were given several groups of rats. These rats were of various ages and body weights. The LD_{50}'s calculated by the methods of Bliss[10] were found to be of the order of 35, 41, 54, and 74 mg per 100 g body weight, respectively for the 4 concentrations given above. These data were criticized on several grounds by Professor J. H. Burn during a visit to this laboratory. Following his suggestions the experiment was repeated in such a way that on any testing day, at least 4 groups of rats of the same body weight were used. For each such 4 groups of rats, 1 group received each of the choline hydrochloride solutions as before. An attempt was made on each test to obtain approximately identical percentage kills from doses of each concentration.

A large series of rats were studied and found to show about the same order of dependence of mortality on concentration as had been obtained in the earlier, less well controlled test. The scatter in the data was so serious that LD_{50} values are given only as ranges (bottom, Table I—combined data from both series). It is clear that the greater the concentration of choline hydrochloride in solution, the less of the drug is required to kill the average rat.

The symptoms of acute choline poisoning have been described in detail elsewhere. A few points bear additional discussion. The lethal effect is extremely rapid following high doses—in many groups of 15 rats receiving such doses all the rats which died had done so within 5 minutes of injection. Changes in respiration in these rats were closely followed by trembling, convulsive movements, salivation, hemorrhage around the eyes in about two-thirds of the rats (Fig. 1) dying from the drug, cyanosis, respiratory paralysis, and death. The dark stain around the eyes was

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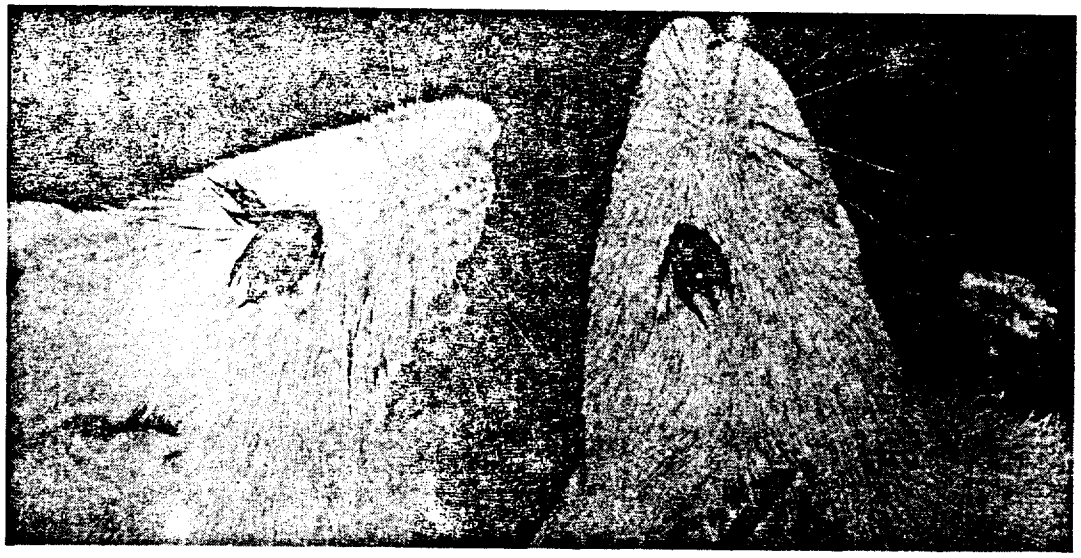

FIG. 1.
Hemorrhage around the eyes after large doses of choline hydrochloride. The bleeding seemed to start from the medial canthus. Excessive salivation may be noted on the lower jaw of rat on the left.

identified as blood by Dr. H. R. Brown, Jr. On first examinations, no red cells were found and we were on the point of concluding, as has Barnard recently,[11] that some porphyrin was responsible. However, more careful and prompt examinations showed the presence of rapidly hemolyzing red blood cells in a saline suspension of the exudate. The saline suspension gave a positive guaiac test. In a stained smear, small red blood cells were found and several polymorphonuclear leucocytes identified. This hemorrhage may be attributed to the intense parasympathomimetic action of overwhelmingly large doses of choline.

Rats which survived 20 minutes after injection of any dose invariably lived. This ability of the organism to excrete or to metabolize and detoxify large amounts of choline has been repeatedly noted. No symptoms of poisoning were evident in surviving rats at any time during an observation period of a week following the various tests.

Summary. 1. The amount of choline hydrochloride to kill the average rat (LD_{50}) is greater the more dilute the solution in which it is injected intraperitoneally.

2. Expressed as mg per 100 g body weight, the LD_{50}'s are as follows: 29-34 for a solution containing 200 mg of choline per ml; 37-38 for a solution of 100 mg per ml; 41-49 for a solution of 40 mg per ml; and 59-75 for a solution of 20 mg per ml.

The author acknowledges the valuable criticisms of Dr. J. H. Burn and wishes to thank Ray Kesel for assistance in handling the animals.

[11] Barnard, R. D., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 254.

Proc. Soc. Exp. Biol. & Med. 58(3):212-215. 1945

Chronic Oral Toxicity of Choline Chloride in Rats.*

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The demonstration of the ability of the rat to metabolize relatively large single doses of choline chloride[1] led to an attempt to discover what limits of tolerance exist for chronic administration. Two series of experiments were therefore carried out, (a) in which choline chloride at various levels was incorporated in the solid diet and (b) in which solutions of choline chloride were substituted for the drinking water.

Choline Chloride in Food. Choline chloride at levels of 0.01, 1, 2, 7, 5, and 10% of the diet was fed for periods of 3 to 4 months to groups of 5 rats each. The average body weight curves (Fig. 1) demonstrated that choline at 0.01 and 1% of the diet produced no effect on growth as compared to litter mate controls. 2.7% of choline reduced growth by about 20%, 5% of choline by about 45%, and 10% of choline in the diet permitted no growth.

Food intakes were measured daily for each group over an 8-week period (Table I., A). The lower growth rates in the groups on higher choline intakes were not a result of any specific toxic effect but were evidence of a refusal of food.

* This work was supported in part by grants from the Carnegie Corporation of New York, from the Nutrition Foundation, and by grant No. 477 from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association.

This paper was presented in part at the meeting of the American Chemical Society, Pittsburgh, September, 1943.

[1] Hodge, H. C., PROC. SOC. EXP. BIOL. AND MED., 1944, 57, 26.

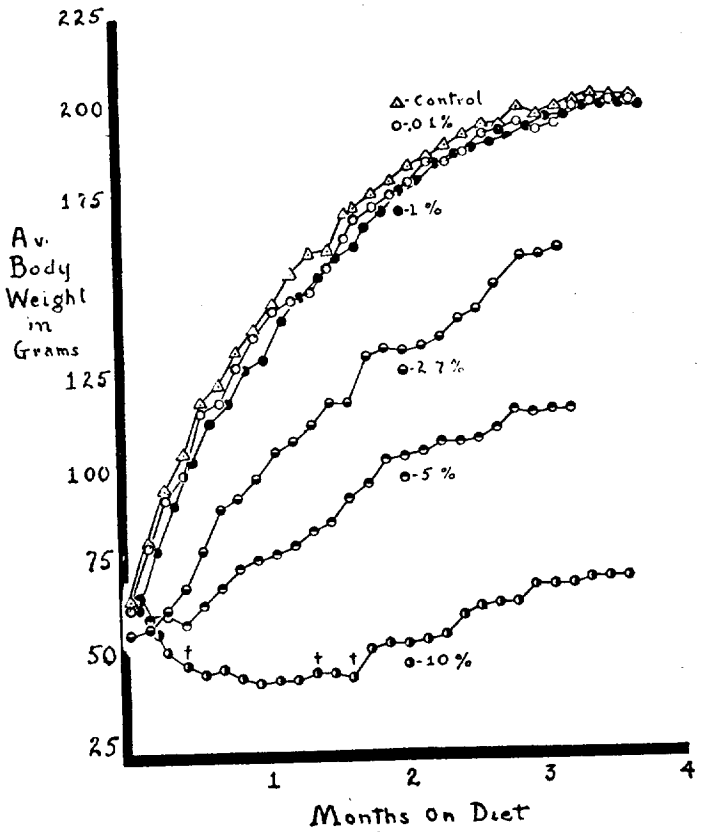

FIG. 1.

Growth curves of groups of rats on various dietary levels of choline chloride. Little effect on growth was observed in diets of 0.01 and 1.0%; progressively greater effects were obtained by including 2.7, 5, and 10% of the drug in the diets. Each + sign marks the death of one rat.

At the end of the experimental period the rats were autopsied. The organs from each rat were weighed and samples of a number of tissues were taken for histological examination. Although the smaller rats (those on the higher choline levels) had smaller organs, the average organ weights per gram rat were comparable for all groups except for the rats whose diet contained 10% choline. In these rats on the highest dosage, the organs (except the spleen) were all much *larger* per gram rat than is normal. In the rats on high choline intake almost no fat was seen either in the subcutaneous tissues, the mesentery, or the perirenal sites. The absence of fat was probably a result of the low food intake; however, the lipotropic action of choline[2] may also have been partly responsible.

The results of the histological examination conducted by Drs. J. R. Carter and H. Kattus of the Department of Pathology were largely negative. In addition to sections of the brain, stomach, kidney, heart, lung, spleen and liver, sections were also prepared of small intestine, large intestine, and ovary or testis. In the group receiving 0.01% of choline, the tissues were essentially normal. In the group receiving 1% of choline, there was a tendency toward an increase in the spleen of hemosiderin-containing phagocytes; this pigment was separately identified by special iron stains. In this group, one rat kidney showed prominent areas of lymphoid infiltration. In the liver of one rat of the group receiving 2.7% of choline, was found an area of focal degeneration. In the other groups including the con-

[2] Best, C. H., and Huntsman, M. E., *J. Physiol.*, 1932, **75**, 405.

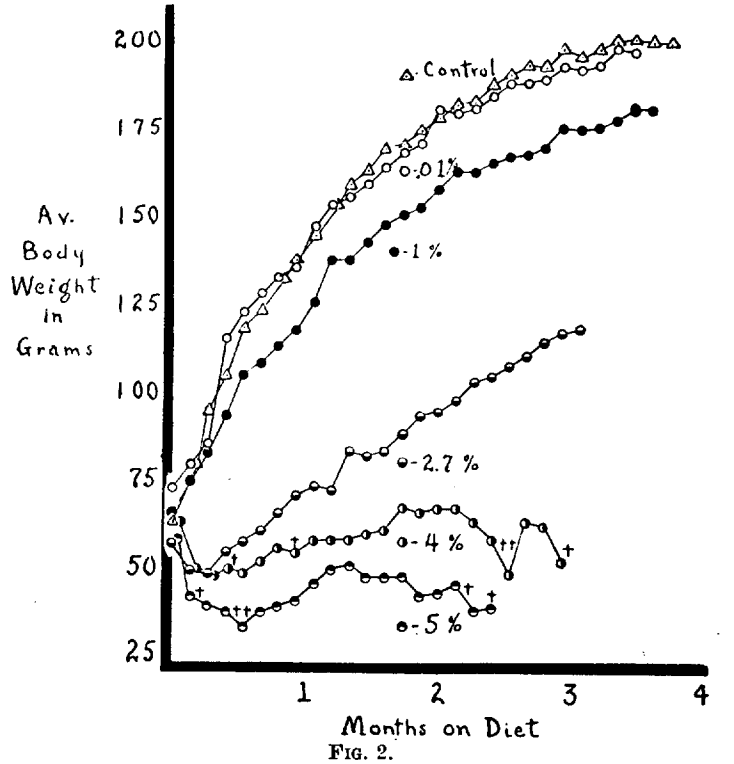

FIG. 2.

Growth curves of groups of rats drinking various solutions of choline chloride. A concentration of 0.01% produced little effect; however, increasing decrements in growth followed drinking 1.0 and 2.7% solutions. Rats given a 5% solution did not regain their original body weight during a period of over 2 months.

trol group, all sections were negative, although (particularly from rats on the higher choline intakes) the sections of many organs appeared smaller than the control tissues. In general, no consistent histopathological changes could be attributed to the inclusion of choline chloride in the food.

Choline Chloride in the Drinking Water. Choline chloride solutions containing 0.01, 1.0, 2.7, 4 and 5% by weight were substituted for the drinking water for the groups of 5 rats each whose growth curves are shown in Fig. 2. The lowest level as was found with the previous dietary experiment produced no diminution of the growth rate. However, drinking 1% choline solution produced a decrease in growth; this was in distinction to the normal growth rate of rats ingesting a 1% choline diet. A 40% reduction in growth was obtained when the rats drank a 2.7% choline solution. No growth occurred when they drank a 4% solution, and on a 5% solution, the survivors after 2 months weighed only 55 and 59%, respectively, of their weight at the start of the experiment. Other groups of rats were given solutions of 0.3, 3.0, 6.7 and 10% choline, respectively. These rats responded as would be expected from the effects seen in the groups described above. No rats drinking 6.7 or 10% choline solutions survived more than about 8 weeks and 7 of 10 rats on these levels died within 10 days.

The water consumption for each group was estimated daily during the first 8 weeks of the experiment (Table I., B). The growth curves reflected the intakes.

At the end of the experimental period the rats were autopsied and the organs treated (Table II., B) as before. The most noticeable consistent change in organ weights occurred in the livers; increasing percentages of choline gave smaller livers. However, when expressed on the basis of percentage of body weight, the average organ weights from the

TABLE I.
Food and Water Consumption by Rats Receiving Various Levels of Choline Chloride.

Choline Content %	A. Choline in diet Avg daily intake Initially g	A. Choline in diet Avg daily intake After 8 weeks g	B. Choline in water Avg daily intake Initially ml	B. Choline in water Avg daily intake After 8 weeks ml
0.01	8.7	12.6	21	33
0.1			14	21
2.7	4.5	12.3	7	12
5.0	3.3	7.3	2	4.5
10	2.1	2.5		

various groups were fairly comparable, except for the group receiving the 3% choline solution. Here the tendency was for the organ weights to constitute larger fractions of the body weight.

The histopathological examinations were carried out as previously described. In general the findings were negative. In the group receiving 0.01% choline, the spleens seemed to show an increase in the hemosiderin-containing phagocytes. In this group, 3 of 5 kidney sections showed in the cortex and pyramids a few focal collections of lymphocytes, most of which appeared to have a peri-arterial distribution. With these few exceptions, for all the other groups including the control rats the tissues were essentially normal. Again it may be said that no consistent pathological findings were observed which might reasonably be attributed to the effect of choline chloride.

Summary. Chronic feeding of a diet containing up to 1% of choline chloride produced no evidence of toxicity. Except for growth rate depression, no toxic effects of higher dietary levels have been observed. Rats which drank water containing 1% of choline chloride showed perceptible diminution in growth rate. Greater concentrations than 3% choline in the drinking water were poorly tolerated. No histopathological effects could be directly attributed to the ingestion of these amounts of choline.

TABLE II.

A. Average Weights of Various Organs of Rats Receiving Diets Containing Various Levels of Choline Chloride. (5 Rats in Each Group)

Choline HCl in diet %	Avg body wt at sacrifice	Average organ weights in grams						
		Brain	Stomach	Kidney	Heart	Lung	Spleen	Liver
0.01	202	1.82	0.98	1.55	0.79	1.35	0.89	6.73
1.0	200	1.64	1.03	1.58	0.70	1.03	0.51	6.58
2.7	162	1.58	1.08	1.18	0.50	0.93	0.64	5.51
5.0	118	1.59	0.90	0.99	0.46	0.63	0.44	3.86
10.0	72	1.38	0.77	0.96	0.43	0.53	0.28	3.12
Control	202	1.59	1.11	1.68	0.62	1.11	0.59	6.54

B. Average Weights of Various Organs of Rats Receiving Various Concentrations of Choline Chloride in the Drinking Water. (5 Rats in Each Group)

Choline HCl in diet %	Avg body wt at sacrifice	Average organ weights in grams						
		Brain	Stomach	Kidney	Heart	Lung	Spleen	Liver
0.01	199	1.65	1.05	1.60	0.67	2.02	0.64	6.27
0.3	165	1.51	0.96	1.55	0.63	0.98	0.50	5.72
1.0	183	1.70	0.90	0.90	0.70	1.10	0.70	5.10
2.7	121	1.50	0.80	1.30	0.50	0.70	0.40	4.10
3.0	76	1.46	0.70	0.98	0.36	0.67	0.25	3.46
Control	188	1.55	0.99	1.52	0.55	1.00	0.70	8.69

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The Acute Toxicity of Choline Hydrochloride in Mice and Rats.*

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Although a number of isolated observations on the acute toxicity of choline have been recorded, no systematic studies are available. The relatively low toxicity of choline is illustrated by the statement of Mott and Halliburton[3] that "We have never succeeded in killing an animal by injection of choline or choline hydrochloride." However, the lethal dose of choline has been estimated for several species as shown in the accompanying text table and the generality has been established that animals either die promptly (within 20 min) from the effects of choline or recover, apparently without permanent injury.

No observations on rats are available.

In the tests reported here, choline hydrochloride (Eastman Kodak Co.) was given in aqueous solutions as indicated (Table I). Death, when it occurred, followed promptly —for large doses within 2-4 min in some of the animals. The symptoms were those previously described by many investigators; in mice, salivation, trembling, jerking, cyanosis, clonic shivering convulsions, respiratory paralysis; in rats, these symptoms with, in addition, a marked bleeding from the eyes which appeared to start from the medial canthus. This hemorrhage was observed in about 60% of the rats dying from the drug.

Toxicity of Choline Hydrochloride.

Species	Lethal dose	Route	Reference
Mice	700 mg/kg	Subcutaneous	1
Cats	35 mg/kg	Intravenous	1,2
Rabbits	1 g/kg	Rectally	5
	0.11 cg/kg	Intravenously	5
	1 g/kg	Subcutaneously	5
Frogs, large	0.1 g	?	4
Frogs, small	0.05 g	?	4

Mice. One hundred six male and female

TABLE I.

Acute Toxicity Data on Choline Hydrochloride for Mice and Rats.

A. Mice—Intraperitoneal Injection of 2% Choline Hydrochloride Solution.

No. Mice	Dose, mg	No. dead	% Mortality
16	5.0	0	0
15	5.6	4	27
15	6.0	7	47
15	6.6	6	40
15	7.0	10	67
15	8.0	13	87
15	10.0	13	87

L.D. 50 (probit kill—5.0) = 6.4 mg per mouse or 320 mg per kg.

B. Rats—Stomach Tube Administration of 67% Choline Hydrochloride Solution.

No. Rats	Dose, g	No. dead	% Mortality
15	.40	0	0
15	.54	3	20
15	.60	3	20
15	.67	6	40
15	.80	9	60
15	1.00	12	80

L.D. 50 (probit kill—5.0) = 0.73 g per rat or 6.7 g per kg.

* This work was supported in part by a grant from the Carnegie Corporation of New York and in part by grant No. 477 from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association.

1 Arai, K., *Arch. ges. Physiol.*, 1922, **193**, 359.

2 Lohmann, A., *Arch. ges. Physiol.*, 1907, **118**, 215.

3 Mott, F. W., and Halliburton, W. D., *Trans. Roy. Soc. Lond.*, B, 1899, **191**, 211.

4 Boehm, R., *Arch. Exp. Path. Pharm.*, 1885, **19**, 87.

5 Dreyfus, L., *C. N. S. B.*, 1920, **83**, 481.

albino mice weighing 18-26 g were given intraperitoneally various doses of choline hydrochloride as a 2% solution in water. No correlation could be observed between body weight (in these limits) and mortality. The fur around the eyes seemed wet to a distance of about 1 mm; the eyes appeared to darken. On the high doses, all the mice that died did so in 4 min after injection. The L.D. 50 (Table IA) was found to be 320 mg per kg by the method of Bliss.[6]

Rats. Ninety male albino rats of average weight about 150 g but varying from 120-200 g were given by stomach tube various doses of choline hydrochloride as a 67% solution in water. No effect on mortality was observed by this variation in body weight. On autopsy, the blood vessels of the diaphragm and stomach were engorged. The liver and spleen appeared congested. Stomachs were bleached and distended. The hearts stopped in diastole. The L.D. 50 (Table IB) was found to be 6.7 g per kg.

Summary. The analyses of the data indicate an L.D. 50 for choline hydrochloride of the order of 320 mg per kg for intraperitoneal injection in albino mice, and of 6.7 g per kg for stomach tube administration in albino rats.

The authors acknowledge the assistance of Ray Kesel.

[6] Bliss, C. I., *Ann. Appl. Biol.*, 1935, **22**, 134.

FURTHER STUDIES ON CHOLINE DEFICIENCY AND MUSCULAR DYSTROPHY IN RABBITS[1]

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(Received for publication April 26, 1957)

A detailed description of choline deficiency in rabbits was given by Hove, Copeland and Salmon ('54) and it was shown by Hove and Copeland ('54) that prolonged deficiency resulted eventually in creatinuria, diminished creatinine excretion, and some hyaline degeneration of striated muscle. Dietary supplements of choline prevented and cured this state of muscular dystrophy as well as other aspects of the deficiency. It is of interest to determine the effectiveness of betaine as a replacement for choline in the diet of rabbits, since the activity of this substance is known to vary with the species of animal under test. For the guinea pig betaine has little or no activity (Reid, '55), while, for lipotropic activity in rats, betaine is completely effective when included in the diet at three times the level of the choline requirement (Young, Lucas, Patterson and Best, '56).

The diet previously used for the production of choline deficiency in rabbits was based upon extracted peanut meal as the main protein source along with a low level of casein. Since methionine had only slight choline-like activity in rabbits (Hove et al., '54), and since Reid ('55) reported that

[1] Supported in part by a grant from the Institute of Neurological Diseases and Blindness (B-430), U. S. P. H. S.

Published with the approval of the Director, Alabama Agricultural Experiment Station. Vitamins used in this study were donated by Merck and Company, Lederle Laboratories, and A. E. Staley Manufacturing Company.

[2] Deceased, June 28, 1957.

J. Nutrition 63(2):289-299. 1957

guinea pigs fed a 20% casein diet developed a choline deficiency characterized by the absence of fatty livers, it was of interest to feed rabbits a casein diet deficient in choline.

The results of these experiments are reported in this paper, together with some data on the effect of choline deficiency on the blood-clotting time and on the quantitative requirement for vitamin E in rabbits. The use of oxidized casein for the production of a methionine deficiency in rabbits is briefly noted.

EXPERIMENTAL

Three choline-deficient diets have been used. These differed in the source of protein and in the level of fat. Diet R36 contained 36% methanol-extracted peanut meal and 6% extracted casein, with 19% lard. Diet R43-C contained 20% extracted casein, with 9% lard. Diet R14E-C contained 40% methanol-extracted soybean meal, with 6% lard. The balance of these diets was composed of 5% salt mixture,[2] 1% potassium bicarbonate, 10% cellulose (non-nutritive fiber), 1% cod-liver oil, and sucrose to 100%. To this was added 0.01% *dl*,α-tocopheryl acetate and the following pure vitamins as micrograms per gram of diet: thiamine, 5; riboflavin, 5; pyridoxine, 5; calcium pantothenate, 25; i-inositol, 200; niacin, 40; methyl, 1–4, naphthoquinone, 0.3; folacin, 2; and vitamin B_{12}, 0.03. The control diets contained 0.12 or 0.20% added choline chloride.

Weanling 4-week-old California-white rabbits were distributed into groups, housed individually in an air-conditioned room on raised, half-inch screens and fed an appropriate diet. They were weighed thrice weekly and the urinary creatine and creatinine were determined three times a week by previously described methods (Hove and Copeland, '54). At death the animals were examined, tissues saved for histological examination by fixing in Bouin's solution and usually stained with hematoxylin-eosin, and the fat content of the oven-dry liver determined by ether extraction overnight.

[2] W. D. Salmon, J. Nutrition, *33:* 155 (1947).

Clotting time of whole blood taken from the marginal ear vein was determined by noting the number of seconds for a drop of blood on a watch glass to develop strands. In most cases this time was determined with and without the addition of a source of "tissue-factor." All determinations were run in triplicate and at 78°F. The source of "tissue-factor" was a 9:1 water-extract of normal rabbit minced lung.

TABLE 1

The effects of betaine and choline on rabbits receiving peanut meal-casein diet

RABBIT NO.	INITIAL BODY WEIGHT	RATE OF GAIN	SURVIVAL TIME	LIVER FAT (dry)	LIVER CIRRHOSIS	URINARY		MUSCLE LESIONS [1]
						Creatine	Creatinine	
	gm	*gm/day*	*days*	*%*	*rating*	*mg/kg/day*	*mg/kg/day*	
(Without supplement)[2]								
122	440	3.4	120	57.3	4	41.0	33.8	mild
123	370	0.6	147	40.0	4	67.8	24.4	severe
125	440	2.8	140	37.7	2	26.0	31.0	mild
220	430	2.7	142 [3]	49.1	4	40.3	26.0	moderate
222	480	4.3	142 [3]	54.8	4	32.4	24.3	. .
Av.:		2.8		47.7		41.5	27.9	
(With 0.3% betaine hydrochloride)[2]								
218	450	14.1	129 [3]	31.8	2	16.2	40.8	trace
219	530	11.5	129 [3]	21.7	3	25.4	36.2	none
221	480	13.4	134 [3]	40.9	2	22.2	40.9	trace
223	410	15.4	125 [3]	24.0	2	15.0	39.7	none
Av.:		13.6		32.1		19.7	39.3	
(With 0.12 choline chloride)								
126	460	21.0	100 [3]	7.9	0	8.6	43.2	none
121	510	21.3	100 [3]	12.8	0	7.0	46.0	none
217	750	22.0	114 [3]	7.9	0	11.2	38.8	none
Av.:		21.4		9.4		8.9	42.7	

[1] Muscle lesions refer to hyaline degeneration in the *femoris triceps*.

[2] All animals on this treatment showed moderate to severe myocardial and endocardial degeneration. Little or no ceroid was present in the cirrhotic livers, and only mildly diffuse bile duct proliferation was seen.

[3] Killed.

RESULTS AND DISCUSSION

Rabbits fed diet R36 (peanut meal + casein), without choline for long periods of time showed poor growth, fatty and cirrhotic livers, high urinary creatine, low urinary creatinine, and histologically defined hyaline degeneration of striated muscle (table 1). Rabbits fed this diet supplemented with 0.3% betaine hydrochloride grew much better, although not at the normal rate, and had normal creatinine and only slightly elevated urinary creatine. On gross inspection the striated muscles of these animals were full-bodied, but had a glassy, translucent, pale greenish-white cast that appeared abnormal. However, no clear evidence of degeneration or abnormality was noted upon histological examination of these tissues. The livers were fatty and cirrhotic; all of the animals in this group had marked cardiac damage characterized as myocardial degeneration, fat in the endocardium, and some valvular degeneration with calcification in a few cases. In spite of the obvious damage to the hearts none of these rabbits died spontaneously.

With 0.12% choline chloride added to the above diet, all animals were normal. The data in table 1 indicate that 0.3% betaine hydrochloride in the diet was too low a level to replace choline completely. Betaine gave effective growth responses and protected striated muscle against histopathology, but it was much less effective than choline in preventing damage to the liver and heart.

Rabbits fed diet R43-C (20% casein) readily developed a choline deficiency (table 2). Only two of the 7 animals fed this diet failed to develop clinical muscular dystrophy (or paralysis), and these two rabbits had died at a relatively early age with marked hydrothorax, ascites, and edema. It is of interest to note that these two rabbits had essentially normal fat in the liver. This is reminiscent of the response of guinea pigs to a similar choline-deficient diet (Reid, '55). Two others of the 7 rabbits had severely fatty livers and cirrhosis; the remainder had moderate cirrhosis and only slightly elevated liver fat. Little correlation existed between the

TABLE 2

Choline deficiency in rabbits fed the 20% casein diet R43-C

RABBIT NO.	INITIAL BODY WEIGHT	RATE OF GAIN	SURVIVAL TIME	GROSS MUSCLE ABNORMALITIES	LIVER FAT (dry basis)	HISTOPATHOLOGY [1]		
						Liver cirrhosis	Muscle lesions	Remarks
	gm	*gm/day*	*days*	*rating*	*%*			
(Without choline)								
264	260	4.7	71	0	11.5	moderate	trace	edema, jaundice, ascites, hydrothorax
265	510	2.3	143 [2]	3	16.9	moderate	mild	incoordination, head retraction
266	310	5.2	170	3	43.1	severe	mild	total hindquarter paralysis, edema
267	390	2.8	60	0	11.2	none	none	edema, ascites, hydrothorax
270	320	4.1	133 [2]	2	20.7	moderate	none	no edema
271	580	3.7	133 [2]	3	50.1	severe	moderate	no edema
274	490	2.6	128 [2]	4	14.6	moderate	none	total paralysis, no edema
(With 0.20% choline chloride)								
268	410	11.3	133 [2]	0	12.3	some atypical fatty changes in liver with occasional zonal necrosis; normal muscle		
269	380	18.2	133 [2]	0	8.9			
272	510	7.9	133 [2]	0	14.8			

[1] Extensive bile duct proliferation accompanied the liver cirrhosis but little or no ceroid was noted. The muscle lesions were hyaline degeneration in the *femoris triceps*.

[2] Killed.

clinical state of the muscular dystrophy or other gross muscle abnormalities, and the histologically demonstrable lesions in the striated muscle. As an example, rabbit 274 was unable to move or to right itself and was classified as grade 4 dystrophy; yet no evidence of hyaline degeneration was noted on histological examination. Urinary creatine was not determined on these animals. Obviously the explanation must be that

TABLE 3

Prolonged blood clotting time in choline-deficient rabbits fed 40% soybran meal diet R14 E-C

RABBIT NO.	INITIAL BODY WEIGHT [1]	BLOOD CLOTTING TIME					LIVER FAT (dry basis) (98 days)
		0 days	21 days	28 days	95 days		
					Direct	With "tissue-factor"	
	kg	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*%*
291	2.25	45	31	37	70	17	18.0
292	2.43	17	33	165	120	16	31.4
293	2.59	34	21	78	125	17	29.2
298	1.31	22	24	93 [2]	30	11	28.0
300	1.65	24	75	120 [2]	20	11	31.4
302	1.89	24	144	137 [2]	44	13	28.0
372 [3]	2.12	18	. .	14	13	9	7.8
373 [3]	1.76	20	. .	17	18	13	6.4

[1] These rabbits had previously been fed a complete casein-basal diet for 66 days from weaning.

[2] Choline supplements of 50 mg/day by dropper started at this time.

[3] Control rabbits fed diet R14E, with choline added at 0.12%.

the paralysis was neurologically induced. However, the brain acetylcholine in such rabbits was lowered to only a slight, and statistically insignificant, extent.[3] On the other hand, the acetylcholine content of brain of rabbits paralyzed due to a vitamin E deficiency was depressed to a significant degree.

It is apparent from the data in table 2 that even with supplementation of choline, the 20% casein diet was not completely adequate for the rabbit; the growth rate of the con-

[3] Hove, E. L., and J. F. Herndon, unpublished data.

trols was not at a maximum level, and some minor liver changes were seen.

Effect of choline deficiency on blood clotting time. A group of 8 control rabbits that had been fed the theoretically com-

TABLE 4

Increased vitamin E requirement in choline-deficient rabbits fed 40% soybean meal diet R14-C or R14

(Initial body weight was 500 to 620 gm)

RABBIT NO.	CHOLINE CHLORIDE IN DIET	TIME TO INITIAL DYSTROPHY	DOSE OF DL, α-TOCOPHERYL ACETATE [1]	BODY WEIGHT AT TIME OF DOSAGE	DAYS CURED OF CREATINURIA	CALCULATED REQUIREMENT FOR VITAMIN E
	%	*days*	*mg*	*kg*	*days*	*mg/kg/day*
1075	0.12	45	20	1.42	19	0.74
			20	1.85	25	0.43
			20	2.13	22	0.43
1073	0.12	26	20	1.44	47	0.30
			20	2.37	27	0.31
1072	0.12	33	20	1.35	35	0.42
			30	1.97	34	0.45
						0.44
Av.:		35				
1071	0	29	20	0.82	17	1.44
			20	1.01	11	1.80
			20	1.12	13	1.37
			20	1.29	10	1.55 (Died)
1069	0	44	20	0.92	21	1.03
			20	0.99	19	1.06
			20	1.13	18	0.99
1068	0	45	20	1.25	26	0.62
			20	1.46	14	0.98
			20	1.62	16	0.77
						1.16
Av.:		39				

[1] These doses were administered when the creatine excretion had reached 80 mg/day.

plete diet R43 (20% casein diet with added choline at 0.2%) was transferred in early adulthood to the 40% soybean meal diet without choline, R14E-C, or to this diet supplemented with choline chloride, diet R14E. Blood clotting time was determined at this point and at intervals thereafter (table 3). After 28 days on the diets, three of the deficient rabbits were supplemented with oral doses of 50 mg choline chloride/day.

Gradual development of a prolonged blood clotting time is apparent from the data in table 3. The clotting time returned nearly to normal in the three rabbits supplemented with choline, even though their livers were still fatty and cirrhotic. The addition of a source of "tissue-factor" resulted in essentially normal clotting times regardless of the severity of the bleeding tendency. These observations indicate that the prolonged clotting time in choline deficiency can not be explained as inadequate absorption of vitamin K from the intestines. Perhaps the explanation may be found in the inadequate formation of the choline-containing phospholipids that make up the "tissue-factor" required in the blood clotting mechanism.

The effect of choline deficiency on the vitamin E requirement. A group of 6 weanling rabbits were fed 40% soybean meal diets with or without choline, diets R14 or R14-C. Both of these diets were deficient in vitamin E, while only one was deficient in choline. The development of muscular dystrophy was followed by measuring the daily creatine excretion. When this had increased to 80 mg/kg body weight, the animals were adjudged dystrophic and were dosed orally with a standard solution of vitamin E (20 mg of *dl*,α-tocopheryl acetate in olive oil was the usual dose). From the body weight at dosage and from the number of days cured of the creatinuria associated with the muscular dystrophy, the values for the vitamin E requirement could be calculated. These are given in table 4. Seven determinations on the three rabbits receiving choline showed an average need of 0.44 mg of *dl*,α-tocopheryl acetate/day/kg body weight. When the rabbits were deficient

in choline, the vitamin E requirement was increased to 1.16 mg of the tocopheryl acetate/day/kg body weight. The increased requirement for vitamin E by choline-deficient rabbits was mentioned by Hove and Copeland ('54).

None of the rabbits on the soybean meal basal diet developed obvious clinical muscle abnormalities due to the prolonged simple choline deficiency. In general, this diet produced less severe choline deficiencies than either of the other two diets used. This was indicated by better growth rates and the absence of spontaneous deaths, as well as by less severe lesions in liver and heart. Perhaps the presence of a better balance of amino acids, such as arginine and glycine or of other less well defined growth factors in the soybean meal, lowered the choline requirement of the rabbit.

Methionine deficiency in rabbits. A methionine deficiency in rabbits was produced by feeding a diet similar to the casein diet R43, except that it contained casein oxidized by the method of Toennies ('42) and supplemented with 0.3% DL-tryptophan. Young rabbits of 400 to 500 gm body weight were used. Two rabbits fed the oxidized-casein diet maintained their body weight for about 50 days after which gradual weight loss began. When these animals died at 68 and 99 days on experiment, their average weight loss was 120 gm. During the last two weeks of life these rabbits developed a paralysis accompained by a creatine excretion as high as 40 mg/day/kg body weight (during the first 50 days the creatine excretion had averaged 5 mg/day). The creatinine excretion averaged 21.0 mg/day/kg. Histologic sections revealed severe hyaline degeneration of striated muscle.

Two other rabbits were fed this diet to which 0.6% methionine had been added. In 83 days these animals had gained 730 gm and were without gross pathology or symptoms when killed. Similarly, two more rabbits were fed the basal oxidized-casein diet to which 0.6% homocystine had been added. This material effectively substituted for methionine, since the average weight gain was 655 gm.

SUMMARY

Choline deficiency in rabbits fed the peanut meal-casein basal diet was characterized by poor growth, early death, fatty and cirrhotic liver, badly damaged heart muscle and valves, high creatine and low creatinine excretion, and hyaline degeneration of striated muscle. Betaine hydrochloride added to the diet at the 0.3% level markedly improved growth, prevented deaths and the muscle damage, but did not prevent heart and liver damage at the level fed. Choline chloride at 0.12% gave complete protection. This diet contained an adequate level of α-tocopherol.

Choline deficiency in rabbits fed the 20% casein basal diet was characterized by edema, hydrothorax, ascites, and early death. About one-half of the cases had approximately normal liver fat and only mild to moderate liver cirrhosis. Incoordination, paralysis, and head retractions were common, but they did not necessarily correlate with the severity of the histopathology of striated muscle.

Choline-deficient rabbits had a prolonged blood clotting time. Choline-deficient rabbits required 1.16 mg *dl*,α-tocopheryl acetate/day/kg, as compared with choline supplemented controls which required 0.44 mg/day/kg. As previously shown, however, 10-fold the normal level of vitamin E will not protect against the muscular dystrophy and creatinuria produced by choline deficiency.

Methionine deficiency in rabbits resulted in moderate weight-loss, death, creatinuria, paralysis and severe hyaline degeneration of striated muscle. Supplements of methionine or of homocystine were equally effective in preventing these deficiency symptoms.

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THE FUNCTION OF CYTIDINE COENZYMES IN THE BIOSYNTHESIS OF PHOSPHOLIPIDES*

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In 1952, Kornberg and Pricer (1) reported in a preliminary communication that P-choline[1] labeled with P^{32} and C^{14} was converted by liver enzymes to a lipide which was not further characterized. The ratio of P^{32} to C^{14} in the product was closely similar to that of the labeled P-choline, suggesting incorporation of both phosphorus and choline as an intact unit into a phospholipide, presumably lecithin. The following reaction mechanism was suggested (2, 3) as a possible explanation for these results:

(1) Phosphatidic acid + phosphorylcholine-P^{32} → lecithin-P^{32} + P_i

Rodbell and Hanahan (4) have studied the incorporation of P-choline into the lipides of isolated mitochondria from the liver of the guinea pig. These workers identified the product of the enzymatic reaction as lecithin, but their results cast little light on possible intermediates.

Although no definite information is available on this point, it appears that a reaction such as Equation 1 should be readily reversible and a phosphorolytic cleavage of lecithin should occur, as a result of which inorganic phosphate labeled with P^{32} should be incorporated into phosphatidic acids. Work in this laboratory on the enzymatic incorporation of P_i^{32} into phospholipides failed to reveal such a reaction. Accordingly, it was decided to investigate the conversion of P-choline to lecithin more closely in an effort to learn the true mechanism of the reaction.

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[1] The following abbreviations will be used in this paper: P-choline = phosphorylcholine; P-ethanolamine = phosphorylethanolamine; ATP = adenosine triphosphate; UTP = uridine-5′-triphosphate; GDP = guanosine-5′-diphosphate; ITP = inosine triphosphate; CTP = cytidine-5′-triphosphate; CDP-choline = cytidine diphosphate choline; CDP-ethanolamine = cytidine diphosphate ethanolamine; UDP-choline = uridine diphosphate choline; GDP-choline = guanosine diphosphate choline; Tris = tris(hydroxymethyl)aminomethane; CoA = coenzyme A; CMP = cytidine-5′-monophosphate; AMP = adenosine-5′-monophosphate; Versene = ethylenediaminetetraacetate; P_i = inorganic orthophosphate; P-O-P = inorganic pyrophosphate.

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In the course of this investigation, a new type of coenzyme, of which the novel compounds cytidine diphosphate choline and cytidine diphosphate ethanolamine are examples, has been found to play an essential rôle in the biosynthesis of phospholipides. This paper will describe the enzymatic synthesis of cytidine diphosphate choline and cytidine diphosphate ethanolamine, the isolation of these compounds from liver and yeast, and their function in the biosynthesis of lecithin and phosphatidylethanolamine.

In 1954, Kennedy (5, 6) reported the incorporation of free choline into the lipides of mitochondria by a pathway not involving P-choline, but requiring ATP and CoA. The enzymatically synthesized lipide was identified as lecithin principally by a procedure which involved the isolation by chromatographic methods of a highly purified lecithin fraction containing alkali-labile radioactive choline. Later work[2] has shown that this identification was erroneous. The actual product is a lipide difficult to distinguish from lecithin by chromatography on alumina, but separable from lecithin on cation exchange resins. It does not contain phosphorus. The suggestion that a separate enzymatic pathway exists for the conversion of free choline into lecithin must therefore be withdrawn. The reactions to be described in the present paper represent the only route for the enzymatic synthesis of phospholipides known at present.

A preliminary account of some aspects of this work has been published (7).

Materials and Methods

A mitochondrial fraction was prepared from the livers of normal adult albino rats by the following procedure. The livers were quickly removed from rats killed by decapitation and homogenized in 5 volumes of ice-cold 0.25 M sucrose containing 0.001 M Versene, in a glass homogenizer of the Potter-Elvehjem type. Nuclei, debris, and whole cells were removed by centrifugation for 5 minutes at 500 × *g* at 0°. The mitochondrial fraction was then obtained by centrifugation at 18,000 × *g* for 20 minutes at 0°, the high speed head of an International refrigerated centrifuge being used, and was washed twice with the sucrose-Versene solution, and finally suspended in sucrose-Versene or water just prior to use. Since it is now recognized that "mitochondria" prepared by conventional procedures of differential centrifugation may represent a range of cytoplasmic granules of varying chemical and biochemical properties (26, 27), this preparation will be described simply as rat liver particles.

The acid phosphatase of human semen was prepared from semen which had been stored at −15° for many months. After thawing, the semina

[2] E. P. Kennedy, unpublished experiments.

plasma was clarified by centrifugation and the protein was precipitated by saturation with ammonium sulfate. The precipitate was removed by centrifugation and dissolved in water (about one-tenth of the volume of the original semen). The enzyme solution was dialyzed against distilled water and then stored at −15°.

Egg lecithin was purified by chromatography on alumina by the method of Hanahan, Turner, and Jayko (8). A mixture of D-α,β-diglycerides was prepared from this egg lecithin by the action of the lecithinase D present in *Clostridium perfringens* type A toxin (9), which was the gift of Dr. H. D. Piersma of the Lederle Laboratories.

Details of the methods for separation of labeled lipides from the enzyme incubation mixture and for the quantitative removal of unchanged labeled substrate have been described by Kennedy (10). In the experiments reported in this paper, carbon tetrachloride rather than ethyl acetate was used when equilibrating the lipide extracts against aqueous buffer. Aliquots of the carbon tetrachloride solution of phospholipides, completely freed of water-soluble radioactive contaminants, were dried in aluminum dishes and counted in a windowless gas flow counter under conditions of negligible self-absorption. The effectiveness of the equilibration procedures was tested by "zero time" controls, in which the trichloroacetic acid was added prior to the enzyme.

Choline-1,2-C^{14} bromide was purchased from Tracerlab, Inc. Ethanolamine-1,2-C^{14} was the gift of Dr. I. Zabin. P-choline and P-ethanolamine were synthesized by a variation of the method of Plimmer and Burch (11). Dipalmitoyl-L-α-glycerophosphoric acid was the gift of Professor E. Baer.

The nucleotides used in this work were products of the Pabst Laboratories, except for the GDP, which was the gift of Dr. D. Sanadi. The amorphous ATP preparation (lot No. 116) was about 95 per cent pure. The crystallized ATP (lot No. 122) was a highly purified product in which no CTP could be detected. The authors wish to express their gratitude to Dr. S. Morell, Dr. A. Frieden, and Dr. S. Lipton of the Pabst Laboratories for generous gifts of several of these nucleotides.

Chromatographic Procedures—Chromatography on Dowex 1 formate (2 per cent cross-linked) ion exchange resin was carried out with an apparatus similar to that described by Busch, Hurlbert, and Potter (12). The mixing chamber initially contained 300 ml. of distilled water. The upper reservoir contained either 0.04 N formic acid (formic acid system) or 0.2 N formic acid adjusted to pH 9.4 by the addition of concentrated ammonium hydroxide (ammonium formate system). Paper chromatography was carried out with the solvents previously described (13).

Other materials and methods were described in previous publications (10, 13).

EXPERIMENTAL

Enzymatic Incorporation of P-choline into Lecithin—When homogenates of rat liver were incubated with P-choline-P^{32} in the presence of $MgCl_2$, phosphate buffer, and a preparation of amorphous ATP (Pabst lot No. 116), a considerable incorporation of radioactivity into phospholipide took place. In confirmation of the work of Kornberg and Pricer (1) and of Rodbell and Hanahan (4), it was found that P-choline-1,2-C^{14} was incorporated at a rate identical with that of P-choline-P^{32}, suggesting that P-choline is incorporated as a unit into the phospholipide structure.

TABLE I

Incorporation of P-choline-P^{32} into Lecithin in Subcellular Fractions of Rat Liver

Tube No.		P-choline incorporated into lipide
		mμmoles
1	Whole homogenate, phosphatidic acid added	5.8
2	" " " " omitted	6.3
3	Particles, phosphatidic acid added	20.0
4	" " " omitted	18.5
5	Supernatant fraction, phosphatidic acid added	2.7
6	" " " " omitted	2.6

Each tube contained 20 μmoles of $MgCl_2$, 100 μmoles of phosphate buffer of pH 7.4, 10 μmoles of P-choline-P^{32} (34,500 c.p.m. per micromole), and enzyme derived from approximately 0.2 gm. wet weight of rat liver. 10 μmoles of amorphous ATP (lot No. 116) were added at the beginning of the experiment, and 1.0 μmole was added every 10 minutes during the course of the reaction. 0.20 μmole of synthetic dipalmitoyl-L-α-glycerophosphate was added as indicated. The final volume of the system was 2.0 ml. The tubes were incubated at 37° for 1 hour.

Further experiments revealed that the particulate fraction of rat liver was considerably more active in the incorporation of P-choline into lipide than the equivalent amount of whole homogenate from which the particles were derived (Table I). For this reason, isolated particles or enzymes derived from particles were used in most subsequent experiments.

The addition of synthetic phosphatidic acid was without effect on the enzyme system.

Identification of Radioactive Product As Lecithin—In the experiments of Kornberg and Pricer (1), the product derived from P-choline was shown to be a lipide and to contain P^{32} and C^{14} in a ratio closely similar to that of the original labeled P-choline, but was not further identified. Rodbell and Hanahan (4) have offered evidence that the lipide enzymatically produced from P-choline is lecithin. Their identification was based on the isolation

of a highly purified radioactive lecithin by chromatography on alumina. These results were confirmed in the present investigation. Since P-choline is incorporated as a unit, the possibility that the actual product might be the phosphorus-free lipide observed by Kennedy[2] to chromatograph with lecithin was excluded. Nevertheless, it was felt desirable to obtain additional proof that the product derived from P-choline was in fact lecithin. This was provided by the isolation of a water-soluble derivative, L-α-glycerophosphorylcholine, from the labeled lipide after mild hydrolysis by the method of Dawson (14). In this procedure, after selective hydrolysis of fatty acids, the water-soluble derivatives of the glycerophosphatides are separated by chromatography on paper. The labeled lipide yielded a single radioactive spot, R_F = 0.84 in the phenol-ammonia solvent of Dawson (14), identical with the R_F of an authentic sample of L-α-glycerophosphorylcholine and closely similar to the value of 0.88 reported by Dawson.

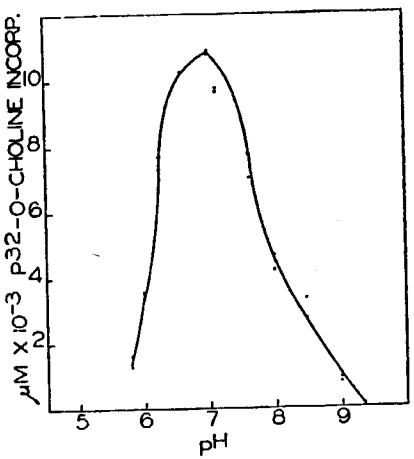

FIG. 1

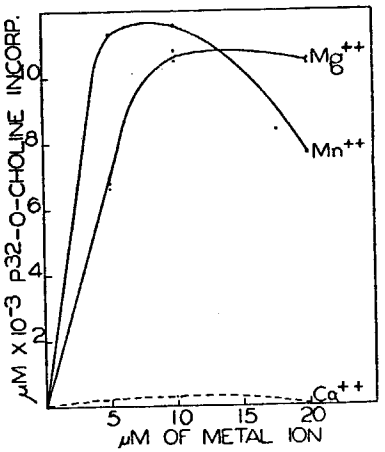

FIG. 2

FIG. 1. Incorporation of P-choline-P^{32} at various pH values. Each tube contained 20 μmoles of $MgCl_2$, 5 μmoles of P-choline-P^{32} (109,000 c.p.m. per micromole), 10 μmoles of ATP (lot No. 116), 1.0 ml. of rat liver particles, and 100 μmoles of an equimolar mixture of phosphate and Tris. The final volume of the system was 2.0 ml. The pH of the Tris-phosphate buffer was varied to give the pH values shown, which were measured with a glass electrode after the addition of all reaction components. The tubes were incubated for 1 hour at 37°.

FIG. 2. The conditions of the enzyme assay were identical with those shown in Fig. 1, except that phosphate buffer of pH 7.4 was used and the added divalent cation concentration varied as indicated.

Optimal pH for Incorporation of P-choline into Lecithin—When the pH of the enzymatic reaction mixture was varied over a considerable range (Fig. 1), the highest incorporation of P-choline took place in the range

6.5 to 7.5. The activity of the enzyme system fell off rapidly at pH values below 6 and above 8.

Requirement for Divalent Cation—The enzymatic conversion of P-choline to lecithin requires the presence of added divalent cations. Manganese is somewhat more effective than magnesium at low concentration in this system, but at higher concentrations it becomes inhibitory (Fig. 2). Calcium is unable to activate the enzyme system; in fact, low concentrations (0.001 M) of calcium ion are inhibitory, even in the presence of optimal amounts of magnesium ion. A similar effect of calcium and barium ions has been reported by Rodbell and Hanahan (4).

TABLE II

Requirement of Amorphous ATP for Incorporation of P-choline-P^{32} into Lecithin

Vessel No.	Energy source	P-choline incorporated into lecithin
		mμmoles
1	Amorphous ATP	20.6
2	Succinate + AMP	0.3

Each vessel contained 20 μmoles of $MgCl_2$, 100 μmoles of phosphate buffer of pH 7.4, 10 μmoles of P-choline-P^{32} (27,500 c.p.m. per micromole), and 1.0 ml. of a suspension of freshly prepared rat liver particles. In the vessels containing amorphous ATP, 10 μmoles of ATP (lot No. 116) were added at the start of the experiment, and 1.0 μmole every 10 minutes during the course of the reaction. To the other vessels, 100 μmoles of sodium succinate + 2 μmoles of AMP were added. The final volume of the system was 2.0 ml. The reaction was carried out in 20 ml. beakers, which were shaken for 1 hour at 37° in a Dubnoff apparatus.

Cofactor Requirement for Conversion of P-choline to Lecithin—No enzymatic conversion of P-choline to lecithin could be observed in experiments with rat liver particles unless ATP (amorphous preparation, Pabst lot No. 116) was added as cofactor. When it was attempted to replace the added ATP with crystalline AMP and an oxidizable substrate (succinate), which could then generate ATP by coupled oxidative phosphorylation *in situ*, no incorporation of P-choline to lecithin took place (Table II). This result was surprising, since earlier work (10) had indicated that coupled oxidative phosphorylation is effective in maintaining a source of metabolic energy for the incorporation of P_i^{32} into phosphatidic acids. It could be demonstrated in control experiments that the oxidative phosphorylation of AMP to ATP was indeed taking place in these experiments.

These results indicated that the actual reaction mechanism by which P-choline is converted to lecithin must be more complex than indicated in Reaction 1.

Rôle of CTP in Conversion of P-choline to Lecithin—When a crystallized

preparation of ATP (lot No. 122) was substituted for the amorphous ATP used in the earlier experiments, no conversion of P-choline to lecithin could be detected. The identity and purity of the ATP lot No. 122 were established by a number of chromatographic, analytical, and enzymatic tests. It was also shown that the ATP lot No. 122 did not contain inhibitors of the enzyme system. It was therefore concluded that some cofactor other than ATP, present in trace amounts in the amorphous ATP lot No. 116, was necessary for the conversion of P-choline to lecithin.

TABLE III

Cofactor Requirement for Incorporation of P-choline-P^{32} into Lecithin

Tube No.	Cofactor added	P-choline incorporated into lecithin
		mμmoles
1	5 μmoles ATP (Lot 116)	5.1
2	5 " " " 122)	0.2
3	5 " " " 122) + 0.5 μmole ITP	0.0
4	5 " " " 122) + 0.5 " UTP	0.4
5	5 " " " 122) + 0.5 " GDP	0.4
6	5 " " " 122) + 0.5 " CTP	15.0
7	5 " " " 122) + 0.5 " " + 2.5 μmoles P-O-P	6.8
8	5 μmoles ATP (Lot 122) + 0.1 μmole CTP	10.0
9	5 " " " 122) + 0.05 " "	6.9
10	5 " " " 122) + 0.1 " CMP	1.1
11	0.5 μmole CTP (no ATP)	8.0

Each tube contained 10 μmoles of $MgCl_2$, 50 μmoles of phosphate buffer of pH 7.4, 3 μmoles of P-choline-P^{32} (113,500 c.p.m. per micromole), and 0.25 ml. of a 10 per cent suspension of lyophilized rat liver particles in a final volume of 1.0 ml. Other cofactors were added as indicated. The tubes were incubated for 1 hour at 37°.

This conclusion also offered an explanation for the experiments in which ATP, formed *in situ* from crystalline AMP by oxidative phosphorylation, was ineffective in supporting the conversion of P-choline to lecithin. The AMP used in these experiments was evidently free of the unidentified cofactor present in ATP lot No. 116.

A number of nucleotides were tested for the ability to replace amorphous ATP in the conversion of P-choline to lecithin. The results of one such experiment are presented in Table III. No significant activity was exhibited by ATP, ITP, UTP, or GDP. It is likely that GDP is at least partially converted to GTP in this system by the action of nucleoside diphosphokinase (15). However, CTP was found to be much more active than the amorphous ATP. It can be seen from the data in Table III that

0.05 μmole of CTP plus 5 μmoles of crystallized ATP is somewhat more effective in promoting the conversion of P-choline to lecithin than 5 μmoles of amorphous ATP, from which it may be concluded that the activity of the amorphous material is due to the presence of less than 1 per cent of CTP as a contaminant.

A slight activating effect is shown by CMP when tested in the presence of ATP. This is due to phosphorylation of the CMP to CTP, as evidenced by the fact that CMP is without activity when tested in the absence of ATP in experiments not shown in Table III. This finding is consistent with the observation that crystallized ATP, completely ineffective in itself, enhances the stimulatory effect of CTP (Table IV), most probably by phosphorylating CMP and CDP to CTP.

Function of Cytidine Coenzymes in Biosynthesis of Lecithin—The discovery of the requirement of CTP for conversion of P-choline to lecithin is the first demonstration of a specific rôle of a cytidine nucleotide in a major metabolic reaction. In Fig. 3 is a simplified scheme postulated to explain the rôle of cytidine nucleotides in the biosynthesis of lecithin.

The essential feature of this scheme is the intermediate formation of the novel compound cytidine diphosphate choline (Fig. 4). The phosphate monoester group of P-choline is converted to a pyrophosphate in the nu-

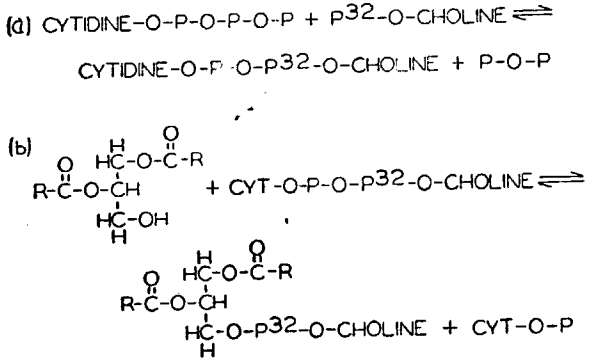

FIG. 3. The function of cytidine nucleotides in the biosynthesis of lecithin

FIG. 4. The structure of cytidine diphosphate choline

cleotide CDP-choline, and is "activated" for a subsequent reaction in which the P-choline moiety is transferred to the free hydroxyl group of a D-α,β-diglyceride. The CMP which is released may then be rephosphorylated to CTP at the expense of ATP in reactions catalyzed by enzymes (15), which are known to have wide-spread occurrence. The cytidine nucleotide thus may act in a continuous catalytic fashion, carrying out a group transfer reaction during the course of which the substrate, P-choline, is built into the structure of the coenzyme itself.

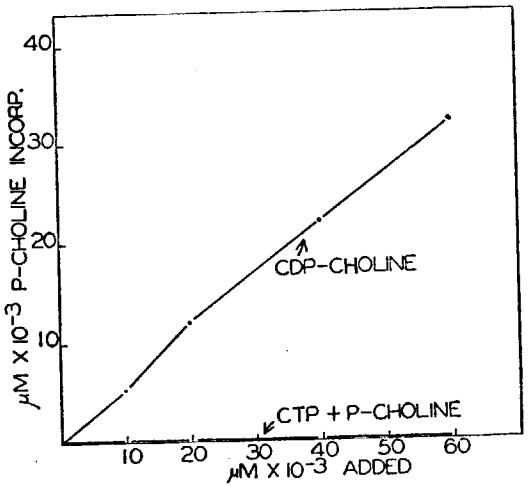

FIG. 5. The enzymatic conversion of synthetic cytidine diphosphate choline to lecithin. Each tube contained 40 μmoles of $MgCl_2$, 100 μmoles of Tris buffer of pH 7.4, 20 μmoles of cysteine, 100 μmoles of KF, and 0.6 ml. of a suspension of rat liver particles in a final volume of 2.0 ml. Varying amounts of CDP-choline (58,000 c.p.m. per micromole) were added as shown. In the control curve, identical amounts of CTP + P-choline-1,2-C^{14} (58,000 c.p.m. per micromole) were added. Incubation was for 90 minutes at 37°.

Three principal lines of experimental evidence have been obtained which offer proof of the formulation shown in Fig. 3. First, highly purified synthetic CDP-choline and CDP-ethanolamine are enzymatically converted to lecithin and phosphatidylethanolamine, respectively, at rapid rates and in high yield. Secondly, CDP-choline and CDP-ethanolamine are naturally occurring nucleotides, which have been isolated from the liver of the rat and the hen, and also from yeast. Finally, enzymes have been found widely distributed in nature which catalyze the reactions shown in Fig. 3.

Enzymatic Conversion of Synthetic CDP-choline to Lecithin—The postulated scheme requires that CDP-choline should be converted to lecithin in the particles enzyme system at a rate at least equal to that of P-choline

plus CTP. The availability of pure synthetic CDP-choline, prepared from P-choline-1,2-C^{14} by the carbodiimide method (13), made it possible to test this point directly. When labeled CDP-choline was incubated with rat liver particles, $MgCl_2$, and buffer, the P-choline moiety was rapidly converted to lecithin (Fig. 5).

It should be noted that the yield of radioactive lecithin from labeled CDP-choline is high (50 to 60 per cent), which strongly suggests that the P-choline of CDP-choline is a direct precursor of the corresponding portion of the lecithin molecule.

The rate and extent of conversion of CDP-choline to lecithin is much greater than that observed with identical amounts of P-choline plus CTP or CMP, a finding which precludes the possibility that CDP-choline is first

TABLE IV

Isotope Dilution Effect of Unlabeled CDP-choline on Incorporation of P-choline into Lecithin

	Radioactivity incorporated into lecithin, total counts
No CDP-choline added	645
1.0 μmole CDP-choline added	17

Each tube contained 5 μmoles of P-choline-1,2-C^{14} (50,000 c.p.m. per micromole), 0.2 μmole of CTP, 10 μmoles of ATP (lot No. 122), 50 μmoles of Tris, pH 7.4, 30 μmoles of KF, and 0.5 ml. of a suspension of rat liver particles. The incubation was for 1 hour at 37°.

hydrolyzed and the breakdown products subsequently incorporated into lecithin.

Isotope Dilution Effect of Unlabeled CDP-choline—If CDP-choline is an obligate intermediate in the reaction sequence by which labeled P-choline is converted to lecithin, then the addition of unlabeled CDP-choline to the reaction mixture should lower the specific activity of the enzymatically generated CDP-choline, and thereby depress the incorporation of radioactivity into lecithin. The experiment in Table IV indicates that the expected isotope dilution effect does occur, supporting the conclusion that CDP-choline is an obligate intermediate in the conversion of P-choline to lecithin.

Enzymatic Conversion of Synthetic CDP-ethanolamine to Phosphatidylethanolamine—The reaction sequence postulated for the biosynthesis of lecithin may, in theory, be readily applied to the biosynthesis of glycerophosphatides in general. The biosynthesis of phosphatidylethanolamine would be expected to involve the intermediary formation of cytidine diphosphate ethanolamine, a compound of structure identical with that of

CDP-choline (Fig. 4), except that ethanolamine replaces the choline portion of the molecule.

Accordingly, CDP-ethanolamine was prepared from P-ethanolamine-P^{32} by the carbodiimide procedure (13) and tested for the ability to act as a precursor of phosphatidylethanolamine. The results of one such experiment are summarized in Table V. It can be seen that the P-ethanolamine portion of CDP-ethanolamine is converted to phosphatidylethanolamine by whole homogenates of rat liver in a yield of about 38 per cent, in a reaction analogous to the formation of lecithin from CDP-choline. When, however, use is made of a preparation of lyophilized isolated particles, which converts CDP-choline to lecithin in good yield, only a slight conversion of CDP-ethanolamine to phosphatidylethanolamine is noted. On the basis of this and other evidence, it appears probable that separate enzymes are required for the formation of lecithin and of phosphatidylethanolamine.

TABLE V

Conversion of CDP-choline and CDP-ethanolamine to Phospholipides

Experiment No.		Radioactivity incorporated into lipide, total counts
1	Whole homogenate, CDP-choline	1485
	" " CDP-ethanolamine	864
2	Lyophilized particles, CDP-choline	1065
	" " CDP-ethanolamine	75

Each tube contained 10 μmoles of $MgCl_2$, 50 μmoles of phosphate buffer of pH 7.4, and 0.5 ml. of either a suspension of lyophilized particles from rat liver or fresh whole homogenate of rat liver. 20 mμmoles of CDP-choline labeled with P-choline-1,2-C^{14} (100,000 c.p.m. per micromole) or 20 mμmoles of CDP-ethanolamine labeled with P-ethanolamine-P^{32} (113,000 c.p.m. per micromole) were added in the tubes indicated. The final volume of the system was 1.0 ml. The tubes were incubated at 37° for 1 hour.

The possibility was considered that the CDP-ethanolamine may be enzymatically converted to CDP-choline before being incorporated into phospholipide. This is not the case, since, after hydrolysis of the enzymatically labeled lipide by the procedure of Dawson (14), glycerophosphorylethanolamine was the only labeled product found. The glycerophosphorylcholine had no detectable radioactivity.

It is to be expected by analogy that CDP-serine might be a precursor of phosphatidylserine. It has not yet been possible to synthesize CDP-serine by the carbodiimide procedure, since the unprotected amino and carboxyl groups of P-serine participate in side reactions leading to a complex mixture of products. It should be possible to synthesize this compound after suitably protecting these functional groups.

Isolation of CDP-choline and CDP-ethanolamine from Liver and Yeast Detectable steady state levels of CDP-choline and CDP-ethanolamine must exist in tissues such as liver which are carrying out an active synthesis of phospholipide, if these compounds are precursors of lecithin and phosphatidylethanolamine *in vivo*. A determination of the concentration of CDP-choline and CDP-ethanolamine in rat liver was made by the following procedure.

Adult female albino rats were decapitated, and the livers removed as quickly as possible and homogenized at once in 10 volumes of 66 per cent ethanol in a Waring blendor at room temperature. A total of 58.9 gm. of rat liver was extracted in this manner, and the extract was clarified by centrifugation. The precipitate was washed twice with a total volume of 200 ml. of 66 per cent ethanol. Small amounts of labeled CDP-choline (0.16 μmole, 16,600 c.p.m.) and CDP-ethanolamine (0.30 μmole, 17,400 c.p.m.) were added to the combined extract and washes. The solution was concentrated to about 250 ml. and extracted with an equal volume of ether. The ether phase was discarded, traces of ether being removed from the aqueous phase by warming slightly under a jet of air. The pH of the solution was adjusted to 8 to 9 with 0.5 N KOH, and the solution passed over a column of Dowex 1 formate (20 cm. high) and chromatographed by using the formic acid system.

Fractions of 11.4 ml. each were collected. The radioactivity was recovered as a single peak in Tubes 21 to 26. No separation of CDP-choline and CDP-ethanolamine was achieved in this system. The material in Tubes 21 to 26 was pooled, neutralized, and rechromatographed by using the ammonium formate system. The volume of each fraction was 16 ml. CDP-choline was recovered in Tubes 6 to 8 and CDP-ethanolamine in Tubes 13 to 19. An unidentified non-radioactive cytidine compound (CDP-X) with 2 atoms of P per mole of cytosine appeared in Tubes 20 to 25.

The isolated nucleotides had the spectrum of pure cytidine compounds. The peaks of radioactivity, due to the small amount of added synthetic radioactive CDP-choline and CDP-ethanolamine, and of absorbancy at 280 μ, due almost entirely to the liver nucleotides, were exactly coincident. The specific activity of successive tubes in each band was constant, indicating the identity and purity of the isolated compounds.

From the specific activity of the added and the reisolated CDP-choline and CDP-ethanolamine, the concentrations of these nucleotides in rat liver may be calculated (Table VI).

The concentrations of CDP-choline and CDP-ethanolamine in rat liver

are sufficiently high so that they may be easily detected without the aid of isotope tracer techniques. Hecht and Potter,[3] in an analysis of the nucleotides of rat liver, using chromatography on Dowex 1 formate, observed the presence of cytidine nucleotides which are eluted with dilute formic acid more rapidly than CMP. These compounds were found to possess 2 atoms of P per mole of cytosine but were not further identified. It is probable that CDP-choline and CDP-ethanolamine are present in these fractions.

Hevesy and Hahn (16) have shown that the phospholipides of the hen's egg are not synthesized in the ovary, but in the liver. The liver of a laying hen must therefore be carrying out a considerable net synthesis of phospholipides, and it was of interest to determine the concentration of CDP-

TABLE VI

Concentration of CDP-choline and CDP-ethanolamine in Liver of Rat and Hen

Experiment No.	Liver	Specific activity		Amount per 100 gm. wet liver
		Added	Recovered	
				μmoles
1	Rat, CDP-choline	104,000	2600	10.4
	" CDP-ethanolamine	58,000	5160	5.3
2	Hen, CDP-choline	50,000	2640	63.0
	" CDP-ethanolamine	58,000	1566	36.0

Details of the experiment with rat liver are given in the text. In the experiment with hen liver, 1.7 μmoles of CDP-choline and 0.50 μmole of CDP-ethanolamine of the specific activities shown were added to the extract derived from 49 gm. of liver.

choline and CDP-ethanolamine in this tissue. The levels of these nucleotides in the liver of the laying hen were found to be about 6 times higher than in the liver of the rat (Table VI). Although a firm conclusion cannot be drawn from the scanty data available, this finding is consistent with the view that the content of CDP-choline and CDP-ethanolamine is high in tissues which are carrying out the biosynthesis of phospholipides at rapid rates.

CDP-choline and CDP-ethanolamine have also been detected in a preparation of crude nucleotides from brewers' yeast (Pabst lot No. X-54). More recently a crystalline nucleotide has been isolated in considerable quantity from yeast by workers at the Sigma Chemical Company. This compound has been conclusively identified as CDP-choline by Dr. I. Lieberman.[4] Samples of pure synthetic CDP-choline and of the crystalline sub-

[3] Personal communication.

[4] Dr. I. Lieberman, personal communication.

stance isolated from yeast have been compared in detail both by Lieberman and in our own laboratory. The compounds are identical in chromatography on ion exchange resins, on paper in several different solvent systems, and in behavior in isolated enzyme systems.

Enzymatic Synthesis of CDP-choline and CDP-ethanolamine—Enzymes have been found widely distributed in nature which catalyze Reaction *a*, Fig. 3, and also the reaction

(2) $$\text{CTP} + \text{P-ethanolamine} \rightleftharpoons \text{CDP-ethanolamine} + \text{P-O-P}$$

In the terminology proposed by Kalckar and Klenow (3), these enzymes may be described as *cytidyl transferases*. Separate enzymes appear to be required for these reactions; it is proposed to refer to the enzyme catalyzing Reaction *a* (Fig. 3) as PC-cytidyl transferase, and to the enzyme catalyzing Equation 2 as PE-cytidyl transferase.

An enzyme extract containing both PC-cytidyl transferase and PE-cytidyl transferase was prepared by the following procedure, all the operations being carried out at 0–5°. 12.5 gm. of rat liver were homogenized for 1 minute in a Waring blendor in 50 ml. of 0.02 M K_2HPO_4 containing 0.001 M Versene. The homogenate was centrifuged at 20,000 × *g* for 40 minutes. The supernatant fluid, containing a considerable amount of particulate material, was diluted to 100 ml. with water, and solid ammonium sulfate was added to 0.40 saturation. The precipitate was collected by centrifugation, suspended in the Tris-Versene solution used for extraction, and dialyzed against the same solution.

The incubation mixture used in the experiments on the enzymatic synthesis of CDP-choline and CDP-ethanolamine contained the following components in a volume of 2.0 ml.: 20 μmoles of $MgCl_2$, 100 μmoles of phosphate buffer, pH 7.4, 10 μmoles of cysteine, 20 μmoles of ATP, 0.4 μmole of CTP, and 20 μmoles of P-choline-P^{32} or P-ethanolamine-P^{32}. 1.0 ml. of enzyme was added, and the tubes were incubated for 1 hour at 37°.

The reaction was stopped by the addition of 5 ml. of 66 per cent ethanol at room temperature. The precipitate of denatured protein was removed by centrifugation and washed with 10 ml. of water. The combined extracts were diluted to a volume of 60 ml. and chromatographed on a column of Dowex 1 formate, the formic acid system being used. The fractions which were collected were then analyzed for radioactivity and for absorbancy at 280 mμ. The results are shown in Fig. 6. In the experiment with P-ethanolamine-P^{32} (lower portion, Fig. 6), the unchanged P-ethanolamine was found in Tubes 11 to 20. About 50 per cent of the added CTP was recovered as CDP-ethanolamine in Tubes 30 to 36. In the experiment with P-choline-P^{32} (upper portion, Fig. 6), about 70 per cent of the CTP was recovered as CDP-choline in Tubes 22 to 29.

The enzymatically synthesized CDP-choline was identical with the synthetic product, as judged by rechromatography on ion exchange resin (ammonium formate system), paper chromatography, absorption spectrum, and biological activity. The enzymatically synthesized CDP-choline was converted to lecithin by rat liver particles in yield identical with that observed with the synthetic product.

Reversibility of PC-cytidyl Transferase—The enzymatic synthesis of CDP-choline shown in Reaction *a* should be readily reversible, since it is closely similar to the formation of DPN from ATP and nicotinamide ribotide which has been found by Kornberg (17) to have an equilibrium con-

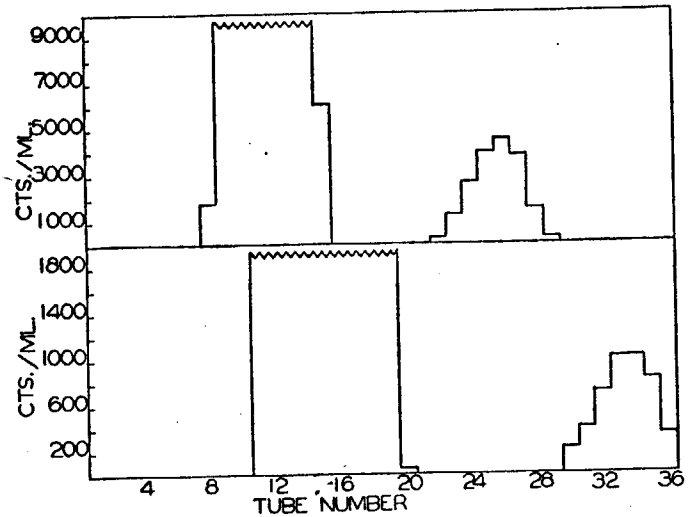

FIG. 6. Chromatographic separation of enzymatically synthesized CDP-choline (upper portion) and CDP-ethanolamine (lower portion). Details of the experiment are given in the text.

stant of about 0.4. PC-cytidyl transferase should therefore catalyze not only the synthesis of CDP-choline from CTP and P-choline, but also pyrophosphorolytic cleavage of CDP-choline to yield CTP and P-choline. In the experiment presented in Table III, the addition of small amounts of pyrophosphate to the enzyme system markedly reduced the over-all incorporation of P-choline into lecithin. This effect was interpreted as indicating that the equilibrium of Reaction *a* had been displaced to the left.

The pyrophosphorolysis of CDP-choline labeled with choline-1,2-C^{14} was studied in the experiment described in Table VII. After incubation for 15 minutes at 37° under the conditions described, the enzymatic reaction was stopped by immersing the tubes in a boiling water bath for 6 minutes. The suspension was centrifuged and 0.50 ml. of the supernatant

fluid assayed for P-choline-1,2-C^{14}. Carrier P-choline (20 μmoles) was added and the pH adjusted to 4.7 with acetate buffer. The P-choline was then quantitatively hydrolyzed to choline by the addition of acid phosphatase from semen, and the choline liberated was recovered as the crystalline reineckate and counted. From the total radioactivity of the choline reineckate and the specific activity of the original CDP-choline, the amount of P-choline formed by the PC-cytidyl transferase reaction could be calculated. The results are given in Table VII. A significant amount of P-choline is formed by the action of purely hydrolytic enzymes, but this value is increased almost 6-fold by the addition of inorganic pyrophosphate, indicating the reversibility of Reaction *a*. Inorganic orthophosphate is without effect on the reaction.

TABLE VII

Pyrophosphorolysis of CDP-choline

Tube No.		CDP-choline split
		mμmoles
1	No pyrophosphate added	23.8
2	5 μmoles pyrophosphate added	123

The enzyme system for the pyrophosphorolytic cleavage of CDP-choline contained the following reagents: 20 μmoles of $MgCl_2$, 50 μmoles of Tris buffer of pH 7.4, 0.6 μmole of CDP-choline labeled with choline-1,2-C^{14} (44,000 c.p.m. per micromole), 5 μmoles of cysteine, and 0.5 ml. of the 0 to 40 ammonium sulfate fraction similar to that used in the experiment in Fig. 6. The tubes were incubated at 37° for 15 minutes.

Distribution of PC-cytidyl and PE-cytidyl Transferases—These enzymes are widely distributed in nature, being found in the liver, brain, heart, and kidney of the rat, in the liver of the guinea pig and hog, in several strains of yeast, and also in carrot root. A detailed study of the properties of the PC-cytidyl transferase of guinea pig liver has been made and will be published in another paper.

PC-glyceride and PE-glyceride Transferases—Reaction *b*, Fig. 3, may be regarded as essentially the transfer of the P-choline portion of CDP-choline to the free hydroxyl group of a D-α,β-diglyceride. The enzyme catalyzing this reaction will be described as PC-glyceride transferase; the analogous but separate enzyme catalyzing the formation of phosphatidylethanolamine from CDP-ethanolamine will be described as PE-glyceride transferase. Evidence that two distinct enzymes are required for these reactions has been given in Table V.

Properties of PC-glyceride Transferase—Added magnesium or manganese ions are necessary for PC-glyceride transferase activity (Fig. 7), just as

for the over-all reaction sequence by which labeled P-choline is incorporated into lecithin (Fig. 2). Calcium ions inhibit PC-glyceride transferase even in the presence of magnesium ions, whereas PC-cytidyl transferase is unaffected by calcium. The inhibition of the over-all system by calcium ions (4) is therefore localized in the PC-glyceride step.

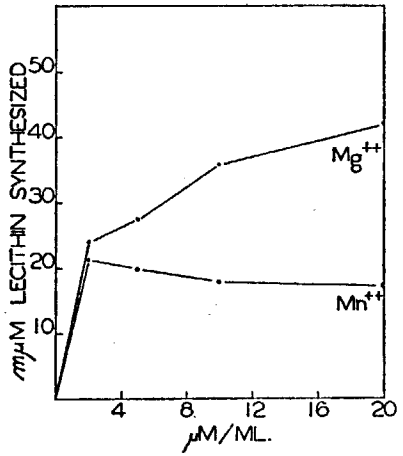

FIG. 7. Requirement of divalent cation for PC-glyceride transferase activity. Each tube contained 0.10 μmole of CDP-choline-1,2-C^{14} (65,000 c.p.m. per micromole), 100 μmoles of phosphate buffer of pH 7.4, and 0.50 ml. of rat liver particles in a final volume of 1.0 ml. Magnesium or manganese ions were added as shown. Incubation was for 1 hour at 37°.

TABLE VIII

Nucleotide Specificity of PC-glyceride Transferase of Rat Liver and Yeast

Tube No.		P-choline incorporated into lecithin, mμmoles	
		Liver enzyme	Yeast enzyme
1	UDP-choline	0.7	0.3
2	GDP-choline	0.0	0.0
3	ADP-choline	0.0	0.0
4	CDP-choline	64.8	84.1

Each tube contained 40 μmoles of $MgCl_2$, 100 μmoles of Tris buffer, pH 7.4, 20 μmoles of cysteine, 100 μmoles of KF, and 0.6 ml. of rat liver particles or crude dialyzed autolysate of brewers' yeast as indicated. 0.20 μmole of labeled nucleotide of identical specific activity (55,000 c.p.m. per micromole) was added as shown. The tubes were incubated for 1 hour at 37°. The final volume was 2.0 ml.

PC-glyceride transferase is highly specific for CDP-choline. When tested with synthetic UDP-choline, GDP-choline, ADP-choline, and CDP-choline (Table VIII), only CDP-choline was found to be active. A similar narrow specificity was found with both the liver and the yeast enzymes.

Requirement for D-α,β-*Diglyceride*—Preparations of particles from the livers of animals of several species convert CDP-choline to lecithin in the absence of added diglyceride. These enzyme preparations are rich in lipide which is bound tenaciously, and the addition of suspensions of lipides of various types suspected to be precursors of the α,β-diglyceride

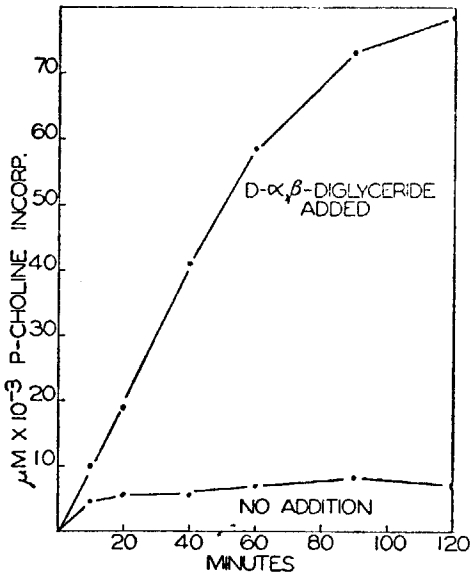

FIG. 8. Requirement of D-α,β-diglyceride for conversion of CDP-choline to lecithin. Each tube contained 20 μmoles of $MgCl_2$, 50 μmoles of Tris buffer of pH 7.4, 0.2 μmole of CDP-choline (58,000 c.p.m. per micromole), 10 μmoles of cysteine, 5 mg. of Tween 20, and 0.2 ml. of rat liver particles in a final volume of 1.0 ml. 3 mg. of D-α,β-diglyceride prepared from purified egg lecithin were added as indicated. The diglyceride was added as an emulsion in the Tween 20; the control received Tween 20 only. The incubation at 37° was varied as shown.

moiety of lecithin was without consistent effect. Similar results were also obtained with enzymes from yeast.

It has been found that surface-active agents, such as bile salts, digitonin, or Tween 20 (polyoxyethylene sorbitan monolaurate), promote the reversible dissociation of α,β-diglyceride from the enzyme surface. Of these, Tween 20 appears to be the most convenient. When rat liver particles are incubated with CDP-choline in the presence of 0.5 per cent Tween 20, the formation of radioactive lecithin is stimulated 10- to 20-fold by the addition of a mixture of D-α,β-diglycerides derived from egg lecithin (Fig. 8). The stimulatory effect of D-α,β-diglycerides is consistent and quite

specific; the egg lecithin from which the D-α,β-diglyceride was derived is much less effective, as is a mixture of naturally occurring triglycerides (corn oil).

DISCUSSION

Workers in several laboratories (10, 14, 18) have observed the presence in a number of tissues of enzyme systems which are active in the synthesis of phosphatidic acids. The concentration of phosphatidic acids in mammalian tissues is so low as to escape detection by ordinary methods. Considerable speculation has arisen as to the physiological significance of this rapid enzymatic synthesis of phosphatidic acids. Evidence has been presented in this paper that D-α,β-diglycerides are precursors of the α,β-diglyceride portion of the lecithin molecule. The dephosphorylation *in vivo* of L-phosphatidic acids would yield D-α,β-diglycerides, which then may be converted to glycerophosphatides. Preliminary experiments in our laboratory have shown that there is a rapid release of inorganic phosphate when phosphatidic acids are incubated with enzymes from rat liver.

The pathways for the enzymatic synthesis of lecithin and phosphatidylethanolamine are essentially similar, suggesting that the mechanisms involved may be applied to the biosynthesis of glycerophosphatides in general, including the serine phosphatides and acetal phospholipides. Sphingomyelin, although not a glycerophosphatide, has a phosphorylcholine moiety like that of lecithin and it is possible that CDP-choline may be a precursor of this portion of the sphingomyelin molecule.

It is noteworthy that the enzymatic reactions in Fig. 3 are widely distributed in nature. Further, enzymes derived from widely different sources, such as liver and yeast, show the same high specificity for cytidine nucleotides. It appears that the fundamental mechanisms involved in the biosynthesis of phospholipides are much the same in many types of living cells.

The brilliant work of Leloir and his collaborators (19–21) has led to the discovery of the uridine coenzymes and of some of the functions of these compounds in the metabolism of carbohydrates. The cytidine compounds described in the present paper have a structure (cytidine-P-P-base) closely similar to the uridine compounds (uridine-P-P-sugar). However, the cytidine coenzymes participate in a type of group transfer reaction which is quite different from any that has been described involving the uridine nucleotides.

The enzymatic synthesis of CDP-choline and of CDP-ethanolamine is similar to the synthesis of uridine diphosphate glucose described by Munch-Petersen *et al.* (22).

(3) UTP + α-glucose-1-phosphate ⇌ uridine diphosphate glucose + P-O-P

Both reactions are examples of a fundamentally important mode of synthesis of nucleotide pyrophosphates discovered by Kornberg in his studies on the biosynthesis of DPN (17).

(4) ATP + nicotinamide ribotide ⇌ DPN + P-O-P

Many of the metabolic transformations of glucose, such as conversion to sucrose (21) or oxidation to glucuronic acid (23), do not occur unless the sugar is first converted to uridine diphosphate glucose. The finding that CDP-choline and CDP-ethanolamine are naturally occurring "activated" forms of choline and ethanolamine raises the question as to whether these compounds undergo reactions other than those leading to the formation of phospholipides. If the analogy with the uridine compounds holds true, then many of the metabolic reactions of choline and ethanolamine may possibly involve CDP-choline or CDP-ethanolamine rather than the free bases themselves.

The presence of adenosine as an essential component of coenzymes has long been known. The work of Leloir and his collaborators on the uridine coenzymes has already been mentioned. Sanadi *et al.* (24) and Kurahashi and Utter (25) have shown that guanosine nucleotides may also participate as cofactors in enzymatic reactions. With the discovery of the cytidine coenzymes, it is now clear that all four of the ribotides which are present in ribonucleic acid are essential components of coenzymes. The underlying biological significance of this fact is not apparent at present, but it must be taken into consideration in any comprehensive theory of the function of ribonucleic acid in living cells.

The authors are indebted to Sylvia Wagner Smith for assistance in many of these experiments.

SUMMARY

1. The enzymatic synthesis of lecithin and of phosphatidylethanolamine has been found to be mediated by cytidine coenzymes. Cytidine diphosphate choline and cytidine diphosphate ethanolamine, "activated" forms of phosphorylcholine and phosphorylethanolamine, are precursors of lecithin and phosphatidylethanolamine, respectively, in these enzyme systems.

2. The levels of cytidine diphosphate choline and cytidine diphosphate ethanolamine have been measured in the livers of the rat and of the hen. These compounds have also been detected in yeast.

3. Enzymes have been found widely distributed in nature which carry out the synthesis of cytidine diphosphate choline and cytidine diphosphate ethanolamine according to the following equations:

CTP + phosphorylcholine $\rightleftharpoons$

cytidine diphosphate choline + inorganic pyrophosphate

CTP + phosphorylethanolamine $\rightleftharpoons$

cytidine diphosphate ethanolamine + inorganic pyrophosphate

The names PC-cytidyl and PE-cytidyl transferases have been suggested for these enzymes.

4. Cytidine diphosphate choline is converted by an enzyme (PC-glyceride transferase) to lecithin, and cytidine diphosphate ethanolamine is converted by a separate enzyme (PE-glyceride transferase) to phosphatidylethanolamine. When tested in the presence of surface-active agents, the PC-glyceride reaction is stimulated 10- to 20-fold by the addition of D-α,β-diglycerides.

5. These results are discussed and a reaction mechanism is presented to account for the function of cytidine coenzymes in the biosynthesis of phospholipides.

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THE EFFECTS OF CERTAIN DIETARY FACTORS ON THE ELIMINATION OF VARIOUS CHLORINATED HYDROCARBONS

[illegible] Franklin FUBENA, Ph.D.
[illegible] University, 1970

Chairman: Dr. C. R. Creger

Two experiments were performed utilizing Japanese quail (Coturnix c. japonica) to determine the effects of the lipotropic agents, choline chloride and inositol, on the elimination of selected chlorinated hydrocarbons from the body.

Supplementation of the diet with 2645 grams of choline chloride and 2000 grams of inositol per 1000 kilograms was effective in accelerating the rate of elimination of the chlorinated hydrocarbons, DDT and heptachlor. The addition of 10 percent vegetable oil to the basal diet increased the quantity of the chlorinated hydrocarbon residues retained in the tissue samples studied.

An increase in liver weight was observed in the groups receiving the diets supplemented with choline chloride and inositol. This indicates increased hepatic activity due to a more rapid mobilization and metabolism of the chlorinated hydrocarbons studied.

Supplementation of the basal diet with choline chloride or inositol singly at levels of 2645 grams or 2000 grams per 1000 kilograms, respectively, was effective in reducing the total quantity of body fat but was not effective in reducing the total carcass residue of DDT and metabolites.

Under these experimental conditions, supplementation of the basal diet of mature laying Japanese quail with 1323 grams of choline chloride and 1000 grams of inositol per 1000 kilograms was as effective as supplementation with 2645 grams of choline chloride and 2000 grams of inositol per 1000 kilograms in accelerating the elimination of DDT and metabolites from the body.

The egg appears to be a very important source for the elimination of chlorinated hydrocarbon residue from the body. The egg yolk is a very reliable indicator of the body tissue residues of the chlorinated hydrocarbons as there was a definite relationship between total carcass residue of DDT and metabolites and egg yolk residue of DDT and metabolites.

Metabolism of the chlorinated hydrocarbons studied appears to occur in the liver. Results of the experiments with heptachlor indicate that, after mobilization and metabolism, elimination of the residue occurs and not deposition in body tissue. This is substantiated by the data from the heptachlor experiments in which only one chromatographic peak emerged upon analysis of muscle tissue samples and adipose tissue samples, whereas three peaks emerged upon analysis of the liver samples.

Under these experimental conditions, the lipotropic agents, choline chloride and inositol, supplemented in combination were effective in reducing total carcass fat content and the total chlorinated hydrocarbon residues present in the birds.

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In 1932, Best et al. /1/ discovered that the choline added to the feed suppressed fat deposition in the liver of a mouse raised on a high-fat, low-protein feed. Later, McHency /2/, and Best et al. /3/ held that choline was essential to the growth of mice and its deficiency retarded their growth. Barnett /4/ observed that the growth retardation due to choline deficiency was notable when the animals were raised on high-fat (30%) ration. With regard to the effect of choline on birds, Jukes /5/ and Hegsted et al. /6/ also noted growth retardation in chicks due to choline deficiency, and asserted that such condition led to perosis or slipped tendon disease. On the other hand, Abbott et al. /7/ observed that the addition of choline to the feed stimulated oviposition and resulted in lower mortality. Foults et al. /8/ reported similar effects of choline on dogs. The author conducted the following growth experiment in an attempt to pursue the relationship between choline and the growth of young mice.

COMPOSITION OF THE FEED

The preparation and purification of the feed followed the procedure described by Torikoshi /9/. The vitamin B's contained in 100 g of feed were: B_1-HCl, 1.0 mg; B_2, 0.4 mg; B_6-HCl, 0.4 mg; pantothenic acid-Ca, 3.0 mg; Biotin, 0.01 mg; inositol, 10.0 mg; PABA, 25.0 mg; nicotinic acid, 1.0 mg.

GROWTH EXPERIMENT

Of young, male DD mice, weighing approximately 8 g, accustomed to a synthetic feed for 4 days, healthy mice were selected and divided into the following 4 groups, each consisting of 10. The animals were raised for 30 days, and weighed at 1 day intervals.

(a) control group
(b) sulfamine group
(c) choline group
(d) choline + sulfamine group

Group (a) was raised on the basal feed without additive, and group (b), on a feed prepared by adding 0.25% of homosulfamine and 0.25% of sulfaguanidine to the basal feed. Group (c) received the basal feed with 400 mg% of choline hydrochloride, and Group (d), the basal feed to which sulfamine and choline hydrochloride were added in the same amounts as in the diet for Group (b) and Group (c), respectively. The feeds were given at 2 g/animal/day up to 10 g of body weight, beyond which the amount was gradually increased up to 3 g. The feeds were combined with water twice the amount of feed, and the mixtures were heated to render pasty consistency.

The growth curves of the individual groups are given in Figures 1 and 2. One mouse of the choline-sulfamine group died during the experiment.

(1) Comparison of Group (a) and Group (c)

Group (a) exhibited a weight gain of 10.8 g in 30 days, from the initial 9.2 g to 20.0 g 30 days later, and Group (c), a weight gain of 11.1 g, from 9.2 g to 20.3 g. The difference in weight gain between the two groups was 0.3 g, which is stochastically not significant. In Group (a), the

hair became increasingly coarse and appeared wet and less dense from the 2nd week, exposing reddish skin at the top of the head.

(2) Comparison between Group (b) and Group (d)

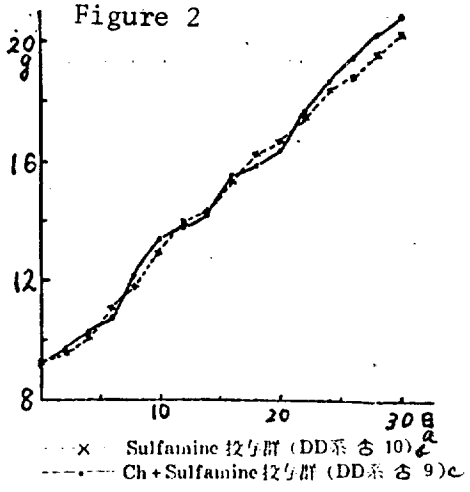
Group (b) showed an average weight gain of 11.1 g, from the initial 9.2 g to 20.3 g 30 days later, and Group (d), an average of 11.7 g, from 9.2 g to 20.9 g. The difference between the two groups is 0.6 g, which is not stochastically significant, with + 0.05. In group (b), the condition of hair was also poor as in Group (a).

CONCLUSION

The effect of choline on the growth of young mice was investigated by raising mice on a synthetic feed supplemented with seemingly sufficient amounts of vitamin B's. At 8 - 12 mg/animal/day, the weight gain attained by the animals during a period of 30 days averaged 10.8 g for the control, 11.1 g for the choline group, 11.1 g for the sulfamine group, and 11.7 g for the sulfamine + choline group. The weight gain shown by the choline group was slightly higher than the control, but the difference was not stochastically significant. However, as compared with the choline group, the control group exhibited poor condition of the hair with coarse texture and moist appearence, exposing reddish skin, the condition pointing to poor growth of the hair.

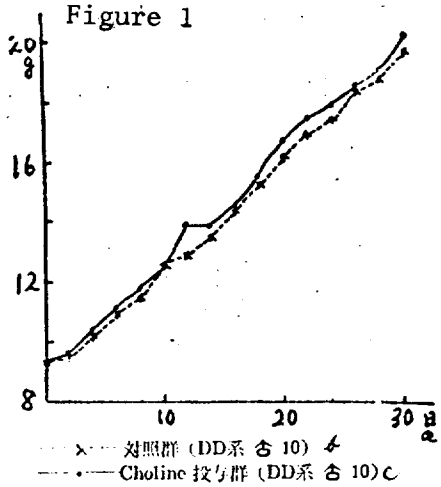
The author is sincerely grateful to Prof. Makino for his guidance and encouragement.

Figure 2



Keys: a, days
b, Sulfamine group (DD; male; 10)
c, Ch + sulfamine group (DD; male; 9)

Figure 1



Keys: a, days
b, control group (DD; male; 10)
c, Choline group (DD; male; 10)

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The Value of Choline Additions to a Corn and Soybean Oil Meal Chick Ration Containing Distillers' Dried Solubles[1,2]

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THE role of choline and methionine in chick nutrition was reviewed in previous papers (Marvel *et al.* 1944, 1945). The development of a modified chemical method for the determination of choline (Engel 1942) has made possible the study of choline deficiency in chick rations. The choline content of some poultry feeds has been reported by Engel (1943), and by Rhian *et al.* (1943). This report compares the choline content of chick rations with the growth responses.

EXPERIMENTAL

The experiments were conducted in the same manner as described in previous reports. Baby chicks were Barred Plymouth Rocks obtained from local hatcheries or hatched from Experiment Station stock. Chicks were sexed by the method of Quinn and Knox (1939), wing-banded, and distributed into lots according to weight.

Approximately 40 chicks were started in each lot, but the numbers were reduced to 25 at four weeks by discarding all chicks except the median chick, and the next 12 on each side of the median. Battery brooders and brooder tables were used. The chicks were fed *ad libitum* on all mash rations. Fresh tap water was supplied daily. The crystalline vitamins* used in the rations were first premixed by dissolving the crystals in 50 percent ethyl alcohol and drying the mixture on ground yellow corn.

The choline content of the ration ingredients used in calculating the choline content of rations is reported in Table 1. The chemical method of Engel (1942) was used to make the determinations.

RESULTS AND DISCUSSION

This station has been concerned with the development of chick broiler rations composed largely of corn and soybean oil meal. When such rations are supplemented with certain vitamins of the B complex, the growth response is comparable to that obtained with practical broiler

[1] Journal paper no. 171 of the Purdue University Agricultural Experiment Station.

[2] This investigation was supported in part by a grant from Joseph E. Seagram & Sons, Inc.

* The choline chloride used in these experiments was generously furnished by the Lederle Laboratories, Pearl River, New York.

TABLE 1.—*Choline content of ration ingredients*

Ingredients	Choline chloride—mg./gm.
Alfalfa leaf meal	1.55
Casein	0.00
Crude lecithin	23.60
Distillers' dried solubles	5.20
Dried skimmilk*	1.59
Ground yellow corn*	0.37
Meat and bone scraps	2.63
Soybean oil meal**	2.80
Wheat bran*	1.43
Wheat shorts*	1.48

* Reported by Engel (1943).
** Six samples—2.77–2.93.

rations containing meat and bone scraps and dried skimmilk. Choline has been shown to be one of the B vitamins necessary for good growth with this type of ration.

Trials I and II.—Two experiments were conducted to show the value of choline additions to the corn and soybean oil meal ration (Table 2). A comparison of the growth responses of lots 1 and 4, trial I, and lots 5 and 7, trial II, shows that the corn, soybean oil meal, alfalfa leaf meal, and distillers' dried solubles

TABLE 2.—*The effect of the addition of choline chloride to a corn and soybean oil meal ration (males)*

Ingredients	Trial I				Trial II			
	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8
Ground yellow corn	52.5	50.0	49.95	49.85	48.0	52.5	52.35	52.0
Soybean oil meal (expeller)	20.0	35.0	35.00	35.00	26.0	34.5	34.50	34.5
Alfalfa leaf meal	5.0	5.0	5.00	5.00	5.0	5.0	5.00	5.0
Distillers' dried solubles		5.0	5.00	5.00		5.0	5.00	5.0
Meat and bone scraps	5.0				5.0			
Dried skimmilk	5.0				5.0			
Wheat bran	5.0				5.0			
Wheat middlings	5.0				5.0			
Riboflavin concentrate[1]		0.5	0.50	0.50		0.5	0.50	0.5
Ca. pantothenate[2]		+	+	+		+	+	+
Pyridoxin hydrochloride[3]		+	+	+		+	+	+
Nicotinic acid[4]		+	+	+		+	+	+
Choline chloride			0.05	0.15			0.15	0.5
Steamed bone meal	1.0	3.0	3.00	3.00		1.5	1.50	1.5
Ground limestone	1.0	1.0	1.00	1.00	0.5	0.5	0.50	0.5
Salt mixture[5]	0.5	0.5	0.50	0.50	0.5	0.5	0.50	0.5
D-activated animal sterol[6]	+	+	+	+	+	+	+	+
Total	100.0	100.0	100.00	100.00	100.0	100.0	100.00	100.0
Percentage Protein	21.0	23.5	23.5	23.5	22.7	22.8	22.8	22.8
Average six-week weights*	306	280	290	351	399	282	367	400
Average eight-week weights*	532	465	504	579	—	—	—	—
Standard deviations[7]	91	154	68	80	73	73	53	82
Coefficients of variation[7]	17.1	33.1	13.5	13.8	18.3	25.9	14.4	20.5
Grams feed per gram gain	2.33	3.02	2.74	2.25	2.23	3.12	2.72	2.15
Number of chicks at end	24	23	23	22	23	24	25	25

[1] 280 micrograms of riboflavin per gram.
[2] 1.95 milligrams per 100 grams of feed.
[3] 400 micrograms per 100 grams of feed.
[4] 30 milligrams per 100 grams of feed.
[5] 95 percent iodized NaCl; 5 percent $MnSO_4$.
[6] 100 units of vitamin D per 100 grams of feed.
[7] Eight-week weights, Trial I.
* Least Significant Difference:
Trial I (eight weeks)—5% level, 62; 1% level, 85.
Trial II (six weeks)—5% level, 39; 1% level, 51.

TABLE 3.—*The effect of additional materials in correcting the apparent choline deficiency (males)*

Ingredients	Lot 9	Lot 10	Lot 11	Lot 12	Lot 13	Lot 14	Lot 15
Ground yellow corn	56.0	55.85	56.0	50.0	61.5	59.0	54.5
Soybean oil meal (expeller)	30.0	30.00		31.0	19.5	24.0	26.5
Autoclaved soybean oil meal			30.0				
Alfalfa leaf meal	5.0	5.00	5.0	5.0	5.0	5.0	5.0
Distillers' dried solubles	5.0	5.00	5.0	5.0	5.0	5.0	10.00
Choline chloride		0.15					
Crude lecithin				5.0			
Domestic casein					5.0		
Meat and bone scraps						5.0	
Riboflavin concentrate[1]	0.5	0.50	0.5	0.5	0.5	0.5	0.5
Ca. pantothenate[2]	+	+	+	+	+	+	+
Pyridoxin hydrochloride[3]	+	+	+	+	+	+	+
Steamed bone meal	2.0	2.00	2.0	2.0	2.0	0.5	2.0
Ground limestone	1.0	1.00	1.0	1.0	1.0	0.5	1.0
Salt mixture[5]	0.5	0.50	0.5	0.5	0.5	0.5	0.5
D-activated animal sterol[6]	+	+	+	+	+	+	+
Total	100.0	100.00	100.0	100.0	100.0	100.0	100.0
Percentage Protein	21.5	21.5	21.5	21.4	21.6	21.5	21.4
Average six-week weights	235	313	245	295	378	373	320
Average eight-week weights	378	435	361	466	583	507	475
Standard deviation*	192	125	131	140	109	102	154
Coefficients of variation*	50.8	28.7	36.3	30.0	18.7	20.1	32.4
Grams feed per gram gain	3.69	3.86	4.21	3.44	2.90	3.28	3.32
Number of chicks at end	22	25	19	20	23	23	21

[1,2,3,5,6] See Table 2.
* Eight-week weights.

rations supplemented with riboflavin, pantothenic acid, pyridoxin, nicotinic acid and choline chloride produced growth as rapid as that obtained from the meat and bone scraps, dried skimmilk practical control rations. However, when the choline was omitted from the corn and soybean oil meal rations, lots 2 and 6, growth was materially reduced. Also, experiments with different levels of choline indicate that growth increased (lots 2, 3 and 4) proportionately as the increments of choline chloride were increased from 0 to 0.15 percent. The same trend was noted in lots 6, 7 and 8. However, previous experiments (Marvel *et al.* 1945) have shown that the growth-promoting value of different samples of soybean oil meal varies, which will affect the requisite amount of choline to be added to a ration for satisfactory growth.

Trial III.—Rations of calculated choline content were fed to the chicks of trial III (Table 3) to compare the growth-promoting value of crystalline choline chloride with the choline contained in natural feed products. A statistical analysis of the differences between the eight-

TABLE 4.—*Differences between the average eight-week weights of the chickens in lots 9 to 15, with statistical significance.*

Lot No.	10	11	12	13	14	15
9	57	17	88*	205**	129**	97*
10	—	74	31	148**	72	40
11	—	—	105*	222**	146**	114**
12	—	—	—	117**	41	9
13	—	—	—	—	76	108*
14	—	—	—	—	—	32

* Least significant difference at the 5% level was 83 gms.
** Least significant difference at the 1% level was 109 gms.

week weights of the chickens in lots 9 to 15 is given in Table 4.

The basal ration, lot 9, produced the usual slow growth. This ration contained approximately 0.14 percent choline chloride. The addition of .15 percent choline chloride, lot 10, making a total of .29 percent choline, resulted in increased growth. When five percent "crude lecithin" was fed, which added approximately 0.12 percent choline, lot 12, growth was comparable to that obtained by the addition of pure choline chloride. This indicates that the choline in lecithin is available to the chick. In an attempt to increase the growth-promoting value of the soybean oil meal by treatment with heat, a process which is known to increase the nutritive value of soybeans, it was found that autoclaving the soybean oil meal did not affect the growth of lot 11. When five percent casein replaced 10.5 percent soybean oil meal, lot 13, which reduced the total choline content of the ration to 0.12 percent, growth was increased to a level much higher than when 0.29 percent choline was present (lot 10). Likewise, when five percent meat and bone scraps replaced six percent soybean oil meal, the total choline content of the ration was lowered to 0.13 percent. Yet the growth response was greater than when the total choline content was 0.29 percent. A similar relationship resulted when an additional five percent of distillers' dried solubles was added to the ration (lot 15), which only increased the total choline content from 0.14 percent to 0.15 percent. Thus, it becomes evident that the total choline content of a ration is of little value in predicting the growth that might be obtained except when crystalline choline chloride is added.

TABLE 5.—*A comparison of the growth-promoting effects of choline and meat and bone scraps (females)*

Ingredients	Lot 16	Lot 17	Lot 18	Lot 19
Ground yellow corn	63.5	62.0	61.8	55.5
Soybean oil meal (expeller)	16.0	22.0	22.0	25.5
Dried skimmilk	5.0	5.0	5.0	
Meat and bone scraps	10.0	5.0	5.0	
Alfalfa leaf meal	5.0	5.0	5.0	5.0
Distiller's dried solubles				10.0
Choline chloride			0.2	1.0
Steamed bone meal				1.5
Ground limestone		0.5	0.5	1.0
Salt mixture[1]	0.5	0.5	0.5	0.5
D-activated animal sterol[2]	+	+	+	+
Total	100.0	100.0	100.0	100.0
Protein	21.0	21.1	21.1	21.0
Average six-week weights	464	478	478	462
Average eight-week weights	721	725	709	704
Standard deviations*	81	67	77	77
Coefficients of variation*	11.2	9.2	10.8	10.4
Grams feed per gram gain	2.58	2.51	2.55	2.46
Number of chicks at end	24	25	24	24

[1] 95 percent NaCl and 5 percent $MnSO_4$
[2] 100 D units per 100 grams feed
* Eight-week weights

Trial IV.—Another experiment sup-

plies additional data regarding the relationship just discussed. One lot of chicks, lot 17 (Table 5), received a ration containing five percent meat and bone scraps. This ration contained only 0.11 percent choline, because dried skimmilk was used as a source of essential B vitamins instead of distillers' dried solubles. If this ration contained a sub-optimal level of choline, the addition of choline chloride should result in increased growth. However, no such response resulted when 0.2 percent of choline chloride was added (lot 18). Furthermore, the addition of five percent more meat and bone scraps did not increase growth (lot 16) above that of lot 17 which received but 5 percent of meat and bone scraps. In lot 19, a ration containing no meat and bone scraps, but containing 10 percent of distillers' dried solubles together with 1.0 percent of choline, gave a growth response comparable to that given by the rations with meat and bone scrap and dried skimmilk. Thus it is seen that when meat and bone scraps, or casein, was included in the ration, no addition of choline was necessary for rapid growth. Yet without additions of choline chloride to rations that did not contain meat and bone scraps, growth was slow. Since the animal protein products supplied little or no choline to the rations containing them, their stimulus to growth could not be explained on the basis of added choline.

An explanation of the growth stimulus of meat and bone scraps and casein on the basis of added methionine in the ration is equally untenable. The five percent casein added to the ration fed lot 13 would add 0.13 percent methionine to this ration, while 0.10 percent methionine was removed when 10.5 percent soybean oil meal was omitted to equilibrate the protein content to that of the ration fed lot 9. Thus there was practically no increase in the total methionine content of the ration fed lot 13 as compared to the ration fed lot 9, yet there was a marked increase in the growth of the chicks receiving the ration containing the casein. The same relationship was true when meat and bone scraps was substituted for some of the soybean oil meal in the ration fed lot 14. Thus, the growth stimulus of casein and meat and bone scraps when added to the simplified corn and soybean oil meal ration cannot be explained on the basis of added choline or methionine to the ration. Therefore, it seems logical to assume that the effect of these additions must be due to differences in availability of either methionine or choline.

SUMMARY

1. The total choline content of chick broiler rations is not a safe index of the adequacy or inadequacy of choline in the ration for growth.

2. Rapid growth was obtained when a basal ration containing corn, soybean oil meal, alfalfa leaf meal, distillers' dried solubles, minerals, and vitamins was supplemented with 0.15 percent choline chloride to give a total choline content of 0.29 percent.

3. At least some of the choline contained in soybean crude lecithin appeared to be available to the chick.

4. Additional heating of a sample of soybean oil meal of inferior growth-promoting value failed to improve the growth-promoting value of the meal.

5. Casein or meat and bone scraps exhibited a choline or methionine-like action, when substituted on an equal protein basis for soybean oil meal in an all-vegetable corn and soybean oil meal chick ration, although these products did not increase the total choline or methionine content of the ration. The effect of these additions may be due to differences in availability of either choline or methio-

nine, or possibly other growth factors.

6. Ten percent of meat and bone scraps was of no more value than five percent meat and bone scraps in the corn and soybean oil meal chick ration.

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14852

Acute Toxicity of Choline Chloride Administered Orally to Rats.*

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From the recent studies on the toxicity of choline chloride in rats and mice[1,2] two pertinent facts may be reiterated: (a) The toxicity increases with increasing concentration when choline chloride solutions are given intraperitoneally to rats, (b) The toxicity following intraperitoneal administration is much higher than that following administration by stomach tube. The following experiments amplify those previously reported[1] on the oral toxicity and show that an increase of toxicity with concentration also occurred when the choline is administered by stomach tube.

Methods. 413 male and female albino rats weighing from 76 to 343 g were given 4 different concentrations of choline chloride solutions.

Groups of 5 to 30 rats each were fasted for 24 hours and then given by stomach tube various doses of each of the 4 concentrations of choline. Several doses of each of the 4 solutions administered on a single day gave fairly consistent results. A roughly linear relationship existed between dosage and percentage mortality. However, on different days marked differences were observed in the susceptibility of the rats; there was considerable scatter in the series as a whole. No explanation for this variation could be found; no correlation existed between sex or weight and toxicity, and the solutions were freshly made for each test.

Results. The results for the 2 higher concentrations (670 and 500 mg/ml) were combined and the results for the 2 lower concentrations (200 and 400 mg/ml) were combined as illustrated in Fig. 1. These values show a significant increase in mortality with increase in concentration. The LD_{50}, calculated by the method of Bliss[3] for the 2 higher concentrations was of the order of 3.4 g/kg body weight. For the 2 lower concentrations, the LD_{50} was of the order of 6.1 g/kg body weight.

The action of choline was rapid; symptoms appeared within 5 to 10 minutes. In most cases, there was an initial excitement period, characterized by jerking movements and occasional convulsions. At this time, the bloody tears (chromodacryorrhea)[4] appeared in 30 to 90% of the animals which succumbed. The excitement period was followed in a few minutes by a depression with complete relaxation and depressed respiration which finally terminated in respiratory paralysis after 15 to 30 minutes. Animals which lived longer than 30 minutes in most cases survived. A few animals which survived several hours were found dead 18 hours after the drug was given. This delayed response was not observed following the intraperitoneal administration of choline.

Summary. 1. 413 albino rats were given solutions of choline chloride by stomach tube. Four concentrations, viz., 670, 500, 400, and 200 mg/ml were used. A significant increase in the toxicity with increase in concentration was found after combining the results for the 2 higher and 2 lower concentrations.

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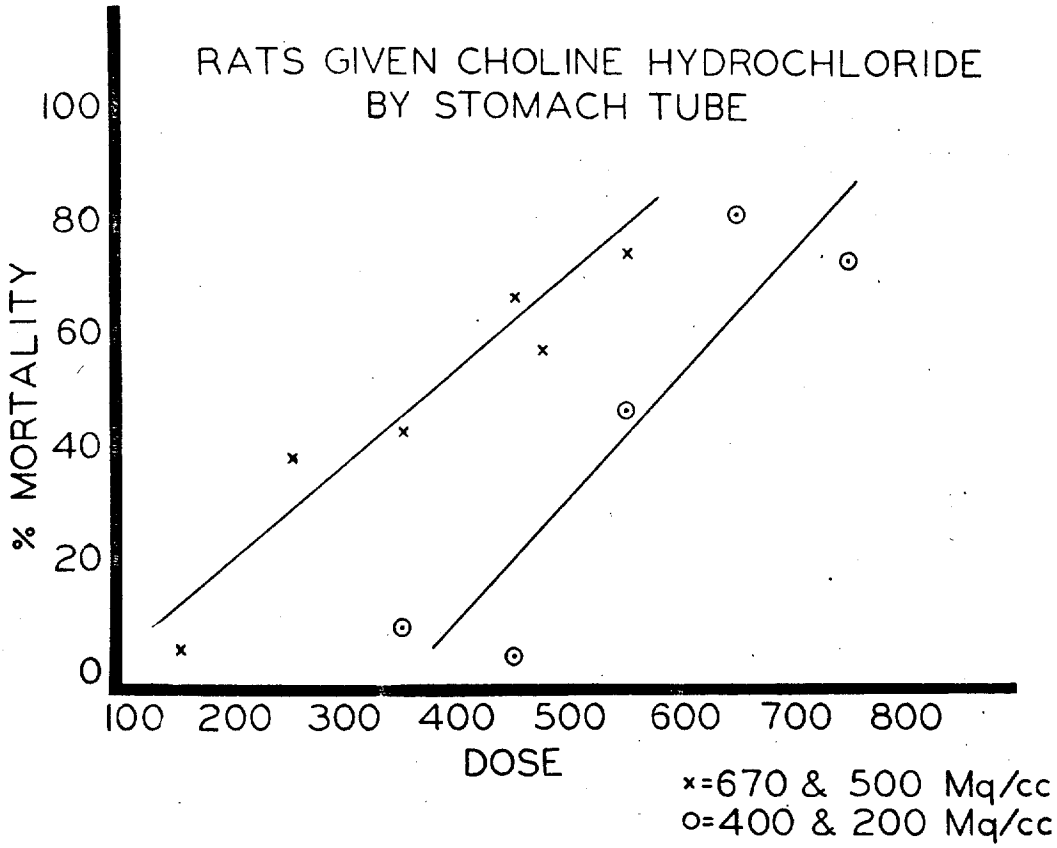

FIG. 1.
These two curves represent the average results obtained with rats given choline solutions containing 670 and 500 mg/ml and 400 and 200 mg/ml, respectively. The dose is represented as mg/100 g rat; expressed as g/kg, the abscissæ are 1.0 to 8.0, respectively.

2. The LD_{50} for the 2 higher concentrations (average 580 mg/ml) was of the order of 3.4 g/kg body weight; for the 2 lower concentrations (average 300 mg/ml) was of the order of 6.1 g/kg body weight.

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The Effect of Varying Amounts of Dietary Cholesterol upon Liver Lipids.

Many investigators studying atherosclerosis and others interested in lipotropic phenomena have fed diets containing cholesterol but no systematic study of the effects on liver lipids of diets containing graded doses of this substance has been reported.

Groups of ten Wistar rats (80 - 120 g.) were fed rations containing varying percentages of cholesterol (0, 0.1, 0.2, 0.4, 0.8 and 1.6) incorporated in a basal hypolipotropic diet consisting of casein 8, gelatin 12, beef dripping 10, corn oil 2, salts 5, cellu flour 2, sucrose 61, with B vitamins and cod liver oil concentrates. Six groups were given corresponding diets containing 0.092% choline chloride (equivalent to 8 mg. choline in 10 g. food). Six groups were given 32 mg. choline and three groups (on the lower doses of cholesterol) were given 32 mg. inositol in 10 g. food.

Total liver lipids on basal diet were 31.8% of net weight; cholesterol (1.6%) increased this to 44.5%; cholesteryl esters increased from 23 to 320 mg. per liver, corresponding to 0.39 and 3.62% respectively of wet weight. Free cholesterol increased slightly (11 to 19 mg. per liver).

When the basal diet contained 0.092% choline chloride total lipids were 8.7%; cholesterol (1.6%) increased this to 19.1%; at the higher level of choline chloride (0.368%) corresponding values were 7.6 and 11.8%. Cholesteryl esters under these four conditions were 5, 213, 4, and 160 mg. per liver, respectively.

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Studies on the Absorption of Choline Chloride*,†

By CARL C. RIEDESEL‡ and H. M. HINES

Two possibilities exist for the metabolism of choline. The present study was undertaken to clarify the manner in which orally ingested choline disappears from the intestine of the rat. It was found that choline disappears from the intestine of the rat at a very constant rate, in a manner suggesting enzymatic absorption. Incubation of choline with intestinal homogenate for the same length of time as required for 39 per cent absorption (two hours) did not result in any decrease of choline concentration. When the intestinal bacteria were suppressed by means of antibiotics in the diet, choline disappeared at the same rate as in the non-treated control rats. It is concluded that choline is absorbed unchanged from the small intestine, and that intestinal bacteria play only a minor role, if any, in its disappearance.

An aspect of the problem of the metabolism of choline concerns its mode of absorption and excretion. De la Huerga and Popper (1, 2) conclude that most of the ingested choline in man is converted to trimethylamine (TMA) or its oxide (TMAO), through the action of bacteria in the small intestine and that these products are then absorbed. Wünsche (3) reports the bacterial decomposition of choline by intestinal bacteria of rats. Norris and Benoit (4) report a similar finding and note that there is a greater excretion of TMAO when choline is given orally to rats than when it is injected intraperitoneally. They explain this phenomenon on the basis of the parenterally introduced choline having had no contact with intestinal flora except for quantities which might conceivably have been excreted into the gut. On the other hand, Toda (5) and Artom and Browder (6) show that liver homogenate and liver slices are able to convert choline to TMA. Thus, while there is ample evidence indicating that choline can be converted to TMA by intestinal bacteria, it is also possible for the liver to accomplish the same task.

The present study was undertaken in an attempt to clarify the manner in which orally ingested choline disappears from the intestine of rats. It was first necessary to measure the rate of disappearance of choline from the small intestine of the rat and to learn if this disappearance could be attributed to bacterial action or to direct absorption. Incubation of choline with intestinal homogenate and determination of the rate of choline disappearance from such a mixture provided one method of ascertaining the significance of the bacterial flora in its metabolism. Another method consisted of inhibiting the bacterial flora with antibiotics and noting any effect on the rate of disappearance of choline from the intestine.

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METHODS AND PROCEDURES

Materials.—All chemicals used were reagent grade. The choline chloride was dried for twenty-four hours at 90°, then stored in a vacuum desiccator over anhydrous calcium chloride. All solutions of choline chloride were made on the day of use, in 0.85% solution of sodium chloride.

The rats used were male, bred from Wistar strain albinos, averaging 150 Gm. in weight. They were maintained on a diet of Purina Laboratory Chow and water, supplied *ad libitum*. Twenty-four hours before any absorption study they were put in wire-bottom cages to prevent access to feces. Water was supplied during this time, but food withheld.

Operative.—For a typical absorption study, the rats were anesthetized with ether. Following a midline abdominal incision, a ligature was put around the small intestine at the site of the ileocecal valve and one just below the entrance of the bile duct. In later experiments, the ligature at the level of the bile duct was discontinued after it had been observed that ligation of the intestine at this position had no noticeable effect on the quantities of choline disappearing from the intestine. Of interest in respect to this latter observation is the report by Rohse and Hines (7) that there is no increase in free choline in the bile of rats following its oral administration.

With the ligature in place at the ileocecal valve, a standard dose of choline chloride was injected into the intestine. For all absorption studies, a 0.1% choline chloride solution was used, the dosage being 200 mg./Kg. of body weight. Injection was made with a 1-ml. tuberculin type syringe and a 25 gauge needle, the injection site being approximately one inch below the entrance of the bile duct. Rate of injection was sixty seconds for the entire dose. Immediately following the choline injection, the abdomen was closed in two layers, and the animals put in a warm cage, protected from drafts to recover from the anesthetic. They were kept in this cage with access to water but not to food. At the end of the respective absorption period, they were quickly killed with ether. The entire small intestine was rapidly removed, and the contents washed into an Erlenmeyer flask by repeated flushing with normal saline at 40°. The total washings were limited to 30 ml.

Analytical.—Proteins were precipitated from solution with a slight modification of the Somogyi method (8). The hydrogen ion concentration of the solution was adjusted to *p*H 10 by addition of a 15% solution of NaOH using Hydrion paper as indicator. Bandelin and Tuschhoff (9) show that such a high *p*H prevents precipitation of contaminants such as the B vitamins and trimethylamine. The solution was filtered by suction, through a double thickness of No. 42 Whatman paper. The precipitate was washed repeatedly with small portions of warm distilled water, the final volume amounting to approximately 150 ml. The choline was precipitated with ammonium reineckate and estimated gravimetrically according to the method described by Pankratz and Bandelin (10).

Separation of Choline from TMA.—Using controlled samples, it was found possible to separate choline from TMA quantitatively by manipulation of the hydrogen-ion concentration of the solvent. Choline is precipitated from aqueous solution by an excess of ammonium reineckate at *p*H 10, while TMA remains in solution. After the choline reineckate is filtered off, adjustment of the hydrogen ion concentration to *p*H 7 will cause immediate precipitation of the TMA reineckate. The TMA can then be gravimetrically estimated.

Incubation of Choline with Gut Homogenate.—Rats which had fasted for twenty-four hours were killed by decapitation and the entire length of the small intestine removed. The excised ileum of each was finely divided in a Waring Blendor and buffered Ringer's solution was used as the dispersion medium. To each separate homogenized ileum was added the same quantity of choline chloride in solution as would have been administered to a live rat for a typical absorption study. The mixture was incubated at 38° for two hours. At the end of this time the proteins were precipitated and gravimetric determinations of choline were made as described under analytical procedure.

Inhibition of Bacterial Flora with Antibiotics.—A diet was prepared containing 0.3% aureomycin and 1% sulfaguanidine in powdered Purina Laboratory Chow. This mixture was supplied to the animals, *ad libitum* for sixty hours. Bacteriological examination of the feces showed complete suppression of the *Coli* organisms but not the *Pseudomonas* organisms. Twenty-four hours before the absorption study, all food was removed from the cage. The absorption study was conducted in the manner described under operative and analytical procedure.

RESULTS

Hourly Absorption Rate.—The hourly absorption rate for the standard solution of choline chloride was determined for periods of one, two, three and four hours. Table I summarizes the data obtained. Results are expressed in mg. of choline chloride absorbed per 100 Gm. body weight.

Incubation of Choline Chloride with Gut Homogenate.—Following the two-hour incubation period, an average of 97.1% of the added choline was recovered. No TMA could be demonstrated. The 2.9% of choline not accounted for may be attributed to experimental error since never was there TMA in sufficient quantity to be estimated gravimetrically. If the amounts of choline chloride which disappeared from the intact intestine in two hours did so because of bacterial action, then incubation of the homogenized intestine with an equivalent amount of choline for the same length of time should show a corresponding rate of disappearance of choline, and a corresponding appearance of TMA. The results are summarized in Table II.

TABLE I.—AMOUNTS OF CHOLINE CHLORIDE ABSORBED PER 100 GM. OF BODY WEIGHT IN THE RAT, DURING ABSORPTION PERIODS OF ONE, TWO, THREE AND FOUR HOURS

Period	Number of Animals	Milligrams Choline Chloride Absorbed per Hour	Percentage Absorption	Total Mg. Absorbed per 100 Gm. Body Weight[a]
1st hour	10	5.61	28.24	5.61
S.D.				1.06
2nd hour	15	3.7	38.98	7.41
S.D.				1.36
3rd hour	13	4.11	63.14	12.34
S.D.				2.79
4th hour	10	4.28	82.74	17.11
S.D.				1.82

[a] In each case the differences between the mean values of total mg. absorbed are statistically significant ($p = < 0.01$).

TABLE II.—EXPERIMENT ON THE RECOVERY OF CHOLINE CHLORIDE ADDED TO INTESTINAL HOMOGENATE AND INCUBATED AT THIRTY-EIGHT DEGREES CENTIGRADE FOR TWO HOURS

Animal Weight	Milligrams Choline Chloride Added	Milligrams Choline Chloride Recovered[a]	Percentage Recovery	Milligrams Trimethylamine Recovered
160	32	30	93.75	00
220	44	43	97.7	00
180	36	36	100.00	00
200	40	40	100.00	00
190	40	38	95.00	00
122	25	24	96.00	00

[a] Average per cent recovery of choline chloride: 97.1.

Intestinal Absorption of Choline Following Inhibition of Intestinal Flora.—During the two-hour absorption period, there was an average disappearance of 41% of the injected choline. The results are summarized in Table III.

TABLE III.—COMPARISON OF THE RATE OF INTESTINAL ABSORPTION OF CHOLINE CHLORIDE BETWEEN NONTREATED CONTROL RATS AND RATS ON A DIET CONTAINING AUREOMYCIN AND SULFAGUANIDINE

Group	Number of Animals	Mean Absorption per 100 Grams in Two Hours[a]
Controls	15	7.4
S.D.		1.36
Treated	11	8.21
S.D.		2.54

[a] The difference between the two mean absorption rates is statistically significant ($p = < 0.01$).

DISCUSSION

The fact that choline disappears from the small intestine of the normal rat at a nearly constant rate of approximately 4 mg./100 Gm. of body weight suggests a process of enzymatic absorption. This is not in agreement with those (1, 2) who believe that bacterial conversion is the prime factor in the disappearance of choline from the small intestine. If bacterial conversion were the prime factor involved

then it should have been possible to show a corresponding rate of disappearance of choline in the incubation studies. However, incubation of choline with finely divided intestine did not bring about any loss of choline other than that which could be attributed to experimental error. Neither was there any measurable quantity of TMA formed during the two-hour incubation period. It is difficult to understand why the same bacteria which *in vivo* could cause a 39% conversion of choline to TMA should not be able to bring about a similar conversion *in vitro* during the same length of time. Furthermore, the administration of aureomycin-sulfaguanidine which was shown to depress the intestinal bacteria did not lower the rate of choline disappearance as it should have done were the bacteria the major factor involved.

There is no evidence to show that the presence of bacteria in the intestine inhibits absorption, or that absorption rates would be increased in the absence of bacteria. One may conclude, therefore, that the absorption of choline would be the same in the presence or absence of intestinal bacteria. Since it was shown that there is a constant rate of disappearance of choline and that this was not inhibited by the antibiotic given, one must also conclude that bacteria play little or no part in the disappearance of choline from the small intestine.

SUMMARY

When choline chloride was administered to rats, in doses of 200 mg./Kg. of body weight, the absorption rate from the small intestine was found to be approximately 4 mg./100 Gm. body weight per hour.

Incubation of choline with intestinal homogenate, for two hours, did not result in any appreciable decrease in choline concentration. When the intestinal bacteria were suppressed, *in vivo*, with a mixture of aureomycin-sulfaguanidine in the diet, choline disappeared from the small intestine at the same rate as in the controls.

It is concluded that choline is absorbed unchanged from the small intestine of the rat, and that the intestinal bacteria play only a minor role, if any, in its disappearance.

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THE EFFECTS OF FEEDING EXCESS DL-METHIONINE AND CHOLINE CHLORIDE TO RATS ON A CASEIN DIET*

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It has been shown by du Vigneaud and coworkers (1–3) that the methyl group as it occurs in methionine, choline, and other sources is a dietary essential. These investigators and others have also shown (4–6) that man and other animals are capable of transferring this methyl group from one compound to another, as, for example, from choline to homocysteine to form methionine, and from methionine to guanidoacetic acid to form creatine. This process is known as transmethylation.

By the use of radioactive tracers (7) it has been proved that the methyl group of methionine may also be oxidized directly to carbon dioxide, as much as one-third of that ingested being oxidized by the rat in 52 hours.

The metabolism of methionine and choline also bears a close and important relationship to fat metabolism, since a lack of either of these dietary essentials will lead to the formation of fatty livers and hemorrhagic kidneys (8–9).

A new method of approach has been to feed excessive amounts of methionine, under which conditions certain striking physiological changes may be detected (10–11). It has been shown (12) with Sherman strain rats that the addition of 4.8 per cent methionine to a 12 per cent casein diet causes a marked loss in weight, a large part of which represents fat stores of the animal. The animals may continue to lose weight while in positive nitrogen balance under these conditions, which may be interpreted to mean that extensive catabolism of fat is continuing. At the same time there is a marked hypertrophy of the kidney.

If the effects observed are due to the increased demands of the body to metabolize methyl groups, either by oxidation or by elimination as the methyl group of creatinine, we might expect that methyl groups of choline, fed in excessive amounts, would tend to cause similar physiological changes. The work described below was designed to test the effects of feeding a high level of choline and to explain further the relationships existing between this compound and methionine.

* This work was done under contract with the Office of Naval Research, Navy Department, Washington, D. C.

EXPERIMENTAL

Five groups of male, Sherman strain rats, weighing approximately 250 gm. each, were utilized. There were ten rats in each group, housed two in a cage. The basal diet consisted of the following ingredients per 1000 gm.: Labco casein 120 gm., sucrose 154 gm., dextrose 222 gm., dextrin 202 gm., lard 252 gm., Wesson's salt mixture (13) 17 gm., and agar 33 gm. To prepare the diet, 1400 gm. of water were utilized per 1000 gm. of solid materials. The agar was first dissolved in two-thirds of the boiling water, and then the lard was added, followed by a slurry of the other ingredients in the remainder of the water. Mechanical stirring was employed to obtain a nearly homogeneous mixture and any losses in water due to evaporation were made up. Just before the diet was ready to solidify, the following vitamin supplement, per kilo of dry diet, was stirred into the mixture: thiamine hydrochloride 2 mg.; pyridoxine hydrochloride 1.6 mg.; calcium pantothenate 40 mg.; niacin 40 mg.; 2-methyl-1,4-naphthoquinone 0.2 mg.; *p*-aminobenzoic acid 40 mg.; inositol 100 mg.; riboflavin 3.2 mg.; biotin 0.2 mg.; folic acid 0.2 mg.; cod liver oil, containing 1800 U. S. P. units of vitamin A and 180 U. S. P. units of vitamin D per gm., 10 gm.; and α-tocopherol 40 mg.

The five groups of rats received the following, in addition to the basic diet, per 1000 gm. of dry ingredients: Group I, 48 gm. (4.8 per cent) of DL-methionine plus 1 gm. of choline chloride; Group II, 70 gm. (7.0 per cent) of DL-methionine plus 1 gm. of choline chloride; Group III, 48 gm. (4.8 per cent) of DL-methionine (no choline chloride); Group IV, 48 gm. (4.8 per cent) of DL-methionine plus 13.5 gm. (1.35 per cent) of choline chloride; and Group V, 13.5 gm. (1.35 per cent) of choline chloride.

The animals in Group I served as controls. The other groups were fed the same weight of diet as was eaten by Group I. Collections of urine and feces were made as previously described (12). The nitrogen intake of Groups I, II, III, and V was approximately the same (Table I). Group IV had a higher intake owing to a higher nitrogen content of the diet. The diet containing 7 per cent DL-methionine (Group II) also had a higher nitrogen content, but the rats on this diet ate less than the controls. In Table II are listed the organ weights of the five groups.

Consideration of the data in Tables I and II shows the following.

The addition of 1.35 per cent choline chloride (Group V) caused no hypertrophy of the kidney, the values for this organ being identical with control values obtained from rats fed the basic diet alone (12). The animals fed excess choline chloride lost considerable weight during the 20 day experimental period, but this also occurs with the basic diet, owing to restriction of food intake. During the last 10 days of the experimental

period the animals fed excess choline chloride were in positive nitrogen balance and gained weight slowly. On the other hand, Sherman strain rats fed 4.8 per cent DL-methionine (Group I), although in positive nitrogen balance during the last 10 days, continued to lose weight slowly. The loss represented fat stores, since the water content of the tissues examined did not change and there was a striking lack of fat in the animal carcass.

TABLE I

Effect of Dietary Supplements of DL-Methionine and Choline Chloride on Nitrogen Balance and Body Weight of Rats during 20 Days

There were five cages of animals in each group. Four 4 day collections were made from each cage and analyses performed in duplicate on each collection. The results were averaged to give the values (twenty values obtained on ten rats in each group) in the table.

Group No.	Supplement		Ingested N	Urinary N	Fecal N	Nitrogen balance	Weight change
	Methionine	Choline chloride					
	per cent	*per cent*	*mg. per kg. per day*	*mg. per kg. per day*	*mg. per kg. per day*	*gm. per kg. per day*	*gm.*
I	4.8	0.1	538	423	82	+0.033	−36.6
II*	7.0	0.1	515	668	74	−0.227	−67.0
III	4.8	0.0	495	493	75	−0.073	−52.4
IV	4.8	1.35	638	550	93	−0.005	−43.3
V	0.0	1.35	515	400	106	+0.009	−33.6

* Sixteen values on eight rats.

TABLE II

Effect of Dietary Sources of DL-Methionine and Choline Chloride on Organ Weights of Rats

The values given are average wet weights per 100 gm. of body weight of ten animals in each group.

Group No.	Supplement		Liver	Kidney	Adrenals	Thyroid	Testes	Seminal vesicles
	Methionine	Choline chloride						
	per cent	*per cent*	*gm.*	*gm.*	*mg.*	*mg.*	*gm.*	*gm.*
I	4.8	0.1	3.15	0.856	12.6	4.5	1.07	0.264
II*	7.0	0.1	2.89	0.900	10.8	4.0	1.15	0.257
III	4.8	0.0	3.04	0.902	14.4	5.1	1.19	0.157
IV	4.8	1.35	3.37	0.860	11.6	4.5	0.89	0.196
V	0.0	1.35	2.87	0.636	11.6	3.9	1.08	0.384

* Average of eight rats.

The absence of choline chloride in the diet containing 4.8 per cent DL-methionine (Group III) aggravated the effects of the methionine. There was a greater loss in weight, a change from a slightly positive to a negative nitrogen balance, and a greater hypertrophy of the kidney. Lack of choline chloride also gave rise to atrophy of the seminal vesicles.

The addition of 1.35 per cent choline chloride to a diet containing 4.8 per cent DL-methionine (Group IV) did not significantly alter the results obtained by feeding 4.8 per cent DL-methionine alone (Group I).

A diet containing 7 per cent DL-methionine (Group II) caused extensive tearing down of the body tissues of the animals, the high negative nitrogen balance continuing at the same levels throughout the 20 day experimental period. Despite this breakdown of body tissue there was a marked hypertrophy of the kidney. There was also an increased excretion of creatine and creatinine under these conditions (Table III).

TABLE III

Creatinine and Creatine Excretion of Rats Fed Excess DL-Methionine

Average excretion for a 20 day period.

Excess methionine fed	Choline fed	Creatinine	Creatine
per cent	*per cent*	*mg. per kg. per day*	*mg. per kg. per day*
7.0	0.1	39.2	6.3
4.8	0.1	28.9	9.4 (12)*
0.0 (Basic diet)	0.1	25.8	5.7 (12)

* The numbers in parentheses refer to bibliographic references.

These last results confirm the reports of Brown and Allison (11) who used rats of the Long-Evans strain. Strain differences are illustrated since the same effects can be obtained in Long-Evans rats with a lower methionine content of the diet. Differences in strains in regard to methionine uptake have also been reported by Rutman and coworkers (14).

DISCUSSION

Excessive quantities of methyl groups, fed in the form of choline chloride, can apparently be metabolized without causing any profound physiological changes in rats. The effects observed when excess DL-methionine is fed may be due, therefore, to the homocysteine portion of the molecule. Du Vigneaud has obtained evidence for this conclusion.[1] The possibility also remains that the intact molecule may be necessary to bring about the observed changes. Hogan (15) has noted that homocystine and choline

[1] du Vigneaud, V., private communication.

when fed together to growing rats, retarded growth to an extent essentially the same as did an equimolar quantity of methionine, but when fed separately they repressed growth only slightly.

Since it has been demonstrated that glycine will, in part, counteract the effects of excess methionine (12, 15), these effects may be the result of excessive demands of the body for extra glycine, or more likely extra serine which may be formed *in vivo* from glycine (16). This is in agreement with the view that one pathway for the metabolism of homocysteine is its conversion to cysteine by combination of homocysteine with serine to form cystathionine (17–19). When the demand for serine may be very high, as when feeding 7 per cent DL-methionine, actual tearing down of body tissues may take place to meet the requirements, this tearing down of tissue causing an increased creatinine excretion. Significantly, the excretion of creatine is not materially higher when excess methionine is fed up to the level of 4.8 per cent in Sherman strain rats, even when extra glycine and arginine are fed in addition (12), conditions which should be expected to favor the formation of creatinine.

The relationship of the metabolism of methionine to fat metabolism remains obscure, but the continued catabolism of fat and loss of fat stores observed in animals in nitrogen balance on a diet containing 4.8 per cent added methionine would seem to indicate that the catabolism of methionine or homocysteine requires the simultaneous catabolism of fat. The report (20) that large doses of methionine fed to humans gave rise to acetone and β-hydroxybutyric acid ketosis lends credence to this conclusion.

SUMMARY

1. A level of 1.35 per cent choline chloride in a diet containing 12 per cent casein is well tolerated by rats of the Sherman strain, and no unusual physiological changes are observed, as occur when excess methyl groups are fed in the form of methionine.

2. The absence of choline chloride in a diet containing 4.8 per cent DL-methionine aggravates the effects of the excess methionine. In addition, atrophy of the seminal vesicles occurs.

3. A level of 7 per cent DL-methionine fed to Sherman strain rats causes extensive tearing down of body tissue, loss in weight, and increase in the excretion of creatinine. A marked hypertrophy of the kidney is also noted.

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Effects of Egg Oil, Cholesterol, Cholic Acid and Choline upon Plasma and Liver Lipids[1]

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ABSTRACT Young adult female rats of the Wistar strain were fed diets containing 27% egg oil or a mixture of butter fat and olive oil with the same iodine number as the egg oil but with different fatty acid content. The other variables were 0.1 or 0.3% choline chloride, 1.0% cholic acid, and 1.29% cholesterol — the level of cholesterol present in the egg oil. The experiments were of 5 to 10 weeks' duration. Rats fed the rations containing the mixture of fats with cholesterol and either level of choline had plasma and liver cholesterol and liver lipid levels that were significantly augmented over those receiving the egg oil. Cholic acid affected an increase in the cholesterol and lipid values of the rats receiving both the fat mixture with cholesterol and the egg oil. The liver lipids and cholesterol decreased when the higher level of choline was fed in conjunction with the mixture of fats with cholesterol and cholic acid. The liver lipids and cholesterol were threefold and twelve-fold, respectively, greater for the rats receiving egg oil than for those fed the fat mixture without cholesterol or cholic acid. Groups given the fat mixture with cholesterol and cholic acid had far higher values than did any of the other groups.

A number of investigators have suggested that certain natural fats contain substance(s) other than cholesterol that increase serum cholesterol. Messinger and co-workers (1) reported that the elevation of serum cholesterol in human subjects fed diets containing egg yolk powder or cream was greater than could be accounted for by the cholesterol contained therein. They concluded, "there is a substance as yet unidentified in egg yolk which elevates the cholesterol level of human serums." Mayer and co-workers (2) failed to note demonstrable change in the plasma cholesterol level when 800 mg of cholesterol/day were given in the form of egg yolk. This observation is in agreement with the results of Keys et al. (3) and Hildreth and co-workers (4). Mayer and co-workers (2) have pointed out that the lack of agreement between the work carried out in their laboratory and that of Messinger et al. (1) could be accounted for by the higher level of egg yolk powder, and the cholesterol contained therein, fed by Messinger et al. The saturated fatty acids in egg yolk could also contribute to the hypercholesterolemia. The cholesterol consumed by Messinger's subjects was at least 4 times that of Mayer's. Bronte-Stewart et al. (5) confirmed the observations of Messinger, giving 10 eggs/day — an amount which supplied essentially the same quantity of cholesterol as received by Messinger's subjects. Portman (6), in a review on factors influencing serum cholesterol levels states: "It appears that the form in which dietary cholesterol occurs is of importance; for example, egg yolk appears to have greater hypercholesteremic activity than does an equivalent amount of crystalline cholesterol. This latter observation may be related to the nonsterol portion of the egg yolk, however. The nature of the basic ration in which the sterol is included may also be of importance."

Wells and Bronte-Stewart (7), working with a single human subject, have recently reported that the cholesterogenic entity in egg yolk resided in the acetone-soluble fraction. Neither the unsaponifiable nor the saponifiable fractions gave an increase in serum cholesterol when fed separately. The 2 fractions were active when fed together but a delay of 6 hours in feeding one or the other nullified this effect. A fat of like iodine number with added cholesterol gave similar effects.

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[1] This study was supported by a grant from the Nutrition Foundation, Inc.

Rowsell et al. (8) fed diets to swine in which 33% of the calories was replaced by egg yolk or by butter fat. Controls received the non-supplemented basal diet. The swine fed egg yolk had approximately 6 times as much aortic atherosclerosis as did the controls, and the butter-fed swine 3 times as much. The group fed egg yolk showed a significant increase in serum cholesterol and phospholipid levels. The swine fed butter fat showed only a slight increase in serum lipid levels.

Hegsted et al. (9) studied the effects of the interaction of cholesterol, cholic acid, and the type of fat on serum cholesterol and vascular sudanophilia in the rat, using 3 levels of cholic acid and 3 levels of cholesterol in all of their combinations. They found that cholic acid had a relatively greater effect than did cholesterol in producing an increase in serum cholesterol. Dose-level responses appeared to indicate an interaction of cholesterol and cholic acid.

The effect of choline on serum cholesterol was studied by Mayfield and Roehm (10) who observed that female rats receiving no choline for 18 days had serum cholesterol values that were essentially the same as those of rats fed varying levels of choline ranging from 30 to 350 mg/100 g diet. Male rats maintained with a choline-free diet had significantly lower serum cholesterol than those receiving the rations supplemented with choline. However, the male and female rats receiving the lower levels of choline — zero, 30 or 60 mg/100 g diet — had lower serum cholesterol when compared with those receiving the higher levels of choline — 100, 250 or 350 mg/100 g diet.

Olson et al. (11) observed hypocholesterolemia, hypolipemia and hypobetalipoproteinemia in rats fed diets deficient in choline and choline-precursors. These effects were prevented by 0.3% choline in the diet and partially prevented by rations containing 18% casein. The level of dietary fat ranging from 6 to 42% and including butter fat, corn oil and lard did not modify the effects of choline upon serum lipids.

The present study deals with the effects of egg oil and of a fat mixture upon plasma cholesterol and liver total lipids and cholesterol of young adult female rats.

MATERIALS AND METHODS

Diets. The purified diets contained 18% casein, 4% salts (Hegsted (12)), optimal vitamins,[2] dextrose and 27% fat (table 1). The level of fat corresponded to the fat content of the average American diet (13). The rations contained either egg oil or a mixture of fats of like iodine number and with or without added cholesterol or cholic acid or both. Choline chloride was fed at 2 levels.

The egg oil was extracted from baker's dried whole egg powder with diethyl ether for 16 hours in a Soxhlet extractor. The residual ether was distilled off; the brown viscous oil that remained was free of peroxides. It was stored at 4°C. The egg oil was analyzed by gas-liquid chromatography for individual fatty acids using a column packing of diethylene glycol succinate on chromosorb W in the Barber-Colman gas chromatograph. The fatty acid composition of the egg oil is shown in table 1. The iodine number as determined by the method of Hanus (14) was 72.

The fat mixture was composed of a 21:6 ratio of olive oil-butter fat. The butter fat was prepared from unsalted butter by heating until molten and centrifuged at 800 rpm for 15 minutes to remove the water and milk solids. The butter fat was frozen until used. A single batch of olive oil stored at 4°C was used. The iodine number for the butter fat was 32 and the olive oil was 85; the resulting mixture of olive oil and butter fat had an iodine number of 73. Although the iodine number indicated a like degree of unsaturation when compared with the egg oil, the content and proportion of individual fatty acids were not the same. The primary difference was in the presence of short-chain fatty acids in the olive oil-butter fat mixture as contrasted with their absence from the egg oil. A higher content of 16:0, 16:1, and 18:2 fatty acids was present in the egg oil.

The fat mixture was fed with and without cholesterol. The fat mixture with added cholesterol contained a total of

[2] Vitamins through the courtesy of Merck, Sharp and Dohme Research Laboratories, Inc., Rahway, New Jersey, and Hoffmann-LaRoche, Inc., Nutley, New Jersey.

TABLE 1

Diet composition

	g/100 g		Iodine no.
Casein	24	Egg oil	72
Fat	27	Olive oil	85
Salts [1]	4	Butter fat	32
Dextrose to	100	Fat mixture	73

Fatty acid composition			Vitamin addendum	
Fatty acids	Egg oil	Fat mixture		
	%	%		mg/100 g
4:0 [2]	—	0.32	Thiamine	1
6:0	—	0.03	Riboflavin	2
8:0	—	0.51	Pyridoxine·HCl	1
10:0	—	0.89	Ca pantothenate	10
10:1	—	0.16	Niacinamide	10
12:0	—	1.31	Inositol	5
14:0	0.03	3.39	*p*-Aminobenzoic acid	30
14:1	—	0.48	Biotin	0.05
16:0	29.50	14.34	Folic acid	0.2
16:1	3.18	1.60	α-Tocopherol	14.2
18:0	9.62	6.12	Vitamin B_{12}	0.01
18:1	47.90	62.88	Menadione	14.2
18:2	9.55	6.61		IU/100 g
19:0	—	1.36	Vitamin A	150
Polyunsaturated	9.55	6.61	Vitamin D	15
Monounsaturated	51.08	65.12		
Saturated	39.42	28.02		

[1] Hegsted et al. (12).
[2] The first figure represents the number of carbon atoms; the second, the number of double bonds.

1.29% cholesterol which corresponded to the cholesterol content of the egg oil as determined by the Sperry-Webb method (15). This quantity was provided by the butter fat containing 0.02% cholesterol and by 1.27% of added cholesterol.

As bile acids are known to increase the absorption of cholesterol, 1% cholic acid was added to some of the diets. Choline chloride was fed as 0.1 or 0.3% of the rations.

The dietary groups were as follows: 1) egg oil with 0.1% choline; 2) egg oil with 0.3% choline; 3) fat mixture with 0.1% choline; 4) fat mixture with 0.3% choline; 5) fat mixture, cholesterol and cholic acid with 0.1% choline; 6) fat mixture, cholesterol and cholic acid with 0.3% choline; 7) fat mixture and cholesterol with 0.1% choline; 8) fat mixture and cholesterol with 0.3% choline; 9) fat mixture and cholic acid with 0.1% choline; 10) fat mixture and cholic acid with 0.3% choline; 11) egg oil and cholic acid with 0.3% choline; and 12) egg oil with 0.3% choline.

This study was carried out in 3 experiments. Experiment 1 (groups 1–6) compared the effects of egg oil with the mixture of fat with and without added cholesterol and cholic acid. Experiment 2 (groups 7–10) compared the individual effects of cholesterol and cholic acid fed with the fat mixture. Experiment 3 (groups 11 and 12) studied the effects of cholic acid upon the absorption of cholesterol as contained in egg oil.

Animals. Young adult female rats of the Wistar strain were used. Groups of 10 rats each were used in experiment 1. The animals averaged 214 g in weight. They were maintained with their respective diets for 10 weeks. Groups of 3 rats each were used in experiment 2. The rats averaged 240 g in weight and were fed the diets for 8 weeks. Groups of 5 rats each were used in experiment 3. They averaged 214 g in weight and the duration of the experiment was 5 weeks.

The rats in all groups were housed in individual cages in an air conditioned room kept at 21.8°C. The diets were fed

ad libitum and food consumption was recorded 3 times weekly. The rats were weighed semi-weekly.

The animals were killed after the period stated by an intraperitoneal injection of pentobarbital sodium.[3] The blood was taken from the deeply anesthetized animals by heart puncture and the livers were removed immediately. The livers were wiped with tissue and weighed. A piece of liver, approximately 2 cm^3 was placed in 30% formalin for histological studies. The remainder was wrapped in Saran Wrap,[4] frozen and stored until used for biochemical determinations.

Methods. Plasma cholesterol was determined by the method of Sperry-Webb (15). The frozen livers were brought to room temperature and ground in a mortar with sodium sulfate. The total lipids were extracted from an aliquot of the wet liver with petroleum ether. An aliquot of the petroleum ether fraction was used for the cholesterol determination (15).

RESULTS AND DISCUSSION

Experiment 1. The food consumption averaged the same for all groups, 12 g daily. The rats receiving the egg oil (groups 1 and 2) made somewhat greater weight gains than those fed the fat mixtures with or without any variants (groups 3–6). The animals made gains expected for females of their age and did not vary appreciably from group to group. All data for experiment 1 are shown in table 2.

The majority of the rats receiving cholesterol and cholic acid with either level of choline (groups 5 and 6) had bloody noses and tails. Several of the animals in these groups excreted urine of gelatinous consistency. The visceral fat in some of these rats was not white and translucent, as in normal animals, but rather brown and opaque in appearance. Despite rough fur and the abnormalities mentioned, the rats were otherwise normal in appearance and activity.

The plasma cholesterol of the rats fed the egg oil diets (groups 1 and 2) was not affected by tripling the choline in their rations. The cholesterol was higher for the animals receiving the egg oil ($P < 0.01$) than for those fed the fat mixture without added cholesterol and cholic acid (groups 3 and 4). The increased choline produced a significant lowering of the cholesterol in the latter groups ($P < 0.05$). The rats receiving the fat mixture with cholic acid and cholesterol (groups 5 and 6) had extremely high levels of plasma cholesterol: namely, a fivefold increase over groups 1 to 4, inclusive ($P < 0.01$). Increasing the choline from 0.1% to 0.3% was without effect upon the plasma cholesterol of groups 5 and 6.

The liver weights were greater for groups 1 and 2 than for groups 3 and 4 ($P < 0.01$). The liver weights of groups 5 and 6 were almost twice those of the other groups. Increasing the choline had no effect.

The total liver lipids and the liver cholesterol were several times higher ($P < 0.01$) when cholesterol and cholic acid were added to the diets containing the fat mixture (groups 5 and 6) than for any other groups. This observation held when calculated on both milligrams per gram and total liver bases. A significant decrease in the liver cholesterol ($P < 0.02$) was produced by the higher level of choline (group 6). The livers from the animals fed diets containing the fat mixture (groups 3 and 4) were of low lipid content and were practically devoid of cholesterol. The liver lipids and cholesterol for the rats fed egg oil (groups 1 and 2) were much higher on both the milligrams per gram and grams per liver bases ($P < 0.01$) than for the corresponding groups given the fat mixture (groups 3 and 4). In the egg oil groups, the increased choline produced a significant decrease in the total lipids ($P < 0.02$), but not in the cholesterol.

Experiment 2. The food consumption averaged the same for all groups — 14 g/day. The rats fed the diets containing cholesterol were somewhat heavier than were those fed cholic acid. One rat in each of the groups receiving cholesterol (groups 7 and 8) had a bloody nose and rough fur. All animals fed the like diet but with cholic acid appeared normal. Data for experiment 2 are given in table 3.

The plasma cholesterol showed almost a threefold increase when cholesterol was

[3] Nembutal, Abbott Laboratories, Inc., North Chicago, Illinois.
[4] Dow Chemical Company, Midland, Michigan.

TABLE 2

Effects of cholesterol, cholic acid and choline on the weights and lipids of rats fed egg oil or a fat mixture

	Weights	Plasma	Liver				
	Final	Cholesterol	Weight	Total lipids		Cholesterol	
	g	*mg/100 ml*	*g*	*mg/g*	*g/liver*	*mg/g*	*g/liver*
Experiment 1, 10 weeks [1]							
1 Egg oil; 0.1% choline	312	102± 2.4 [2]	11.9±0.75	207±11.5	2.50±0.19	43.4±2.3	0.48±0.05
2 Egg oil; 0.3% choline	298	104± 4.3	11.3±0.35	166± 8.5	1.95±0.16	40.6±2.4	0.47±0.04
3 Fat mixture; 0.1% choline	301	88± 7.5	8.9±0.35	66± 3.8	0.57±0.03	3.1±0.3	0.03±0.00
4 Fat mixture; 0.3% choline	286	66± 4.8	7.8±0.71	64± 2.0	0.51±0.02	4.2±0.4	0.03±0.00
5 Fat mixture; 0.1% choline cholesterol; cholic acid	292	471±60.9	20.6±0.71	355± 9.0	7.30±0.37	164.4±5.4	3.57±0.13
6 Fat mixture; 0.3% choline cholesterol; cholic acid	290	547±57.6	20.0±0.71	344± 9.8	6.90±0.40	143.6±4.8	2.66±0.20

[1] Each group consisted of 10 female rats with an average initial weight of 214 g.
[2] SD of mean.

TABLE 3

Effects of cholesterol, cholic acid and choline on the weights and lipids of rats fed egg oil or a fat mixture

	Weights	Plasma	Liver				
	Final	Cholesterol	Weight	Total lipids		Cholesterol	
	g	*mg %*	*g*	*mg/g*	*g/liver*	*mg/g*	*g/liver*
Experiment 2, 8 weeks [1]							
7 Fat mixture; 0.1% choline cholesterol	302	273± 9.5 [2]	9.7±0.72	329± 0.7	3.43±0.02	113.9± 5.9	1.19±0.01
8 Fat mixture; 0.3% choline cholesterol	288	297±32.2	13.0±1.6	294± 4.5	4.26±0.36	63.9±17.3	0.77±0.05
9 Fat mixture; 0.1% choline cholic acid	265	95± 8.7	9.3±0.14	120±17.5	1.10±0.04	17.6± 4.5	0.16±0.02
10 Fat mixture; 0.3% choline cholic acid	272	103±15.2	9.7±0.61	91±11.5	0.86±0.06	13.0± 4.6	0.12±0.01
Experiment 3, 5 weeks [3]							
11 Egg oil; 0.3% choline cholic acid	264	209±31.3	16.3±0.46	185± 7.5	3.02±0.17	50.3± 1.9	0.82±0.03
12 Egg oil; 0.3% choline	287	123± 8.8	14.1±0.76	170± 8.5	2.38±0.14	25.3± 5.5	0.39±0.04

[1] Each group consisted of 3 female rats with an average initial weight of 240 g.
[2] SD of mean.
[3] Each group consisted of 5 female rats with an average initial weight of 214 g.

in the rations (groups 7 and 8) than when cholic acid-containing diets were fed (groups 9 and 10), ($P < 0.01$). The plasma cholesterol was not affected by the choline intakes.

The liver weights appeared to be increased by the higher level of choline in the presence of cholesterol (groups 7 and 8); however, this increment was not significant because of the variance and small number of animals in these groups. Increased choline with cholic acid was without effect (groups 9 and 10).

The total liver lipids and cholesterol followed the same pattern as did the plasma cholesterol in that a threefold increase was noted for the rats fed cholesterol (groups 7 and 8) over those receiving cholic acid (groups 9 and 10). The values for these latter groups (groups 9 and 10) were lower than for any other groups in experiments 1 and 2 except those receiving only the fat mixture (groups 3 and 4). Increasing the choline in the presence of cholesterol produced a significant rise ($P < 0.05$) in the lipids on a gram per liver basis, but not on a milligram per gram basis (groups 7 and 8). The cholesterol did not follow this pattern on a total weight basis; in fact, a lower value was noted. Due to the variance in the small number of observations for groups 9 and 10 the choline appeared to be without significance.

The fat mixture with cholesterol produced a two- to threefold increase in plasma and liver cholesterol and liver total lipids over that observed with the egg oil in experiment 1. The modest increase in food intakes in experiment 2 would not appear to account for this difference.

Experiment 3. The food consumption for the 2 groups in this experiment was higher than in experiment 1 in which egg oil was fed even though the initial weights were the same; the food intake being approximately 20 g/day as compared with 12 g in experiment 1. The weight gains were somewhat less for the animals receiving cholic acid than for those without it (groups 11 and 12). Both groups were in good condition throughout the 5-week experiment. The data for experiment 3 are shown in table 3.

Cholic acid produced an increase of approximately 50% in plasma ($P < 0.05$) and liver cholesterol ($P < 0.01$) and a 20% increment in liver lipids based on grams per liver ($P < 0.02$) over the group not receiving cholic acid. These observations suggest the need for a study of egg lipids as influenced by cholic acid in other species including man.

HISTOLOGY — (Experiments 1 and 2)

The livers from the animals receiving cholesterol and cholic acid (groups 5 and 6) were brownish yellow in color and had a butter-like consistency.

Histological examination revealed that 7 out of the 10 animals receiving the diets containing egg oil (groups 1 and 2) had needle-like crystals, presumably cholesterol, in the cytoplasm. The nuclei of the peri-central zone was atrophic and the cells appeared somewhat atrophied. Increasing the level of choline (group 2) appeared to have little or no effect upon the livers. The values for the liver cholesterol were the same for these groups, an observation noted in experiment 1. Rats that were fed cholesterol and cholic acid with the fat mixture (groups 5 and 6) showed massive fatty depositions with innumerable crystals in the parenchymal cells. The liver cords were somewhat distorted due to the great increase in cell size; in fact, the cell appeared to be completely replaced by fat with little evidence of cytoplasmic remnants. The nuclei were atrophic. Increasing the choline in the diet appeared to have little effect, although slightly less lipid and crystalline deposition was noted.

When cholesterol alone was given in the diet containing 0.1% choline (group 7) a massive crystalline deposition with atrophy of the nuclei was noted. These changes were not as marked as in group 8 in which 0.3% choline was given. The livers of the rats fed the fat mixture without cholesterol and cholic acid (groups 3 and 4) showed only mild fatty deposits which were primarily periportal. Increasing the choline was without effect upon these livers. When cholic acid was given with the fat mixture with either level of choline (groups 9 and 10) the fatty deposits were the least observed for any of

the groups. There were few crystals and only a small number of periportal hepatic parenchymal cells. The choline level was without effect.

The livers from experiment 3 were not examined.

The feeding of egg oil with or without cholic acid resulted in lower plasma and liver cholesterol values in rats than did a mixture of fats with the same level of cholesterol present in egg oil. The results appear to indicate that factors, or a balance of factors, present in egg oil tended to lower the cholesterol levels that would have been expected from the feeding of cholesterol as contained in the egg oil. This observation may hold only for the Wistar rat and would not necessarily be in conflict with the observations of Messinger et al. (11) and Bronte-Stewart et al. (5) who used human subjects. Furthermore, egg oil was fed in our experiments; Messinger used egg yolk powder and Bronte-Stewart fed fresh eggs.

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Relation Between Size of Dose and Lipotropic Effect of Choline Chloride in Mice.*

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In recent years Best and his coworkers have established the effectiveness of choline chloride in preventing (or curing) fatty infiltration of the liver in depancreatized dogs and in rats on a low choline-high fat diet. Preliminary results with mice, chickens and puppies were reported by Best, Huntsman and Solandt,[1] but the choice of animals for choline studies has been restricted essentially to the rat and depancreatized dog.

We have found the mouse an excellent species for the study of the lipotropic action of choline chloride and related compounds, in many ways preferable to the rat. It is possible to complete an experiment satisfactorily in 5 or 6 days, while it has been the custom to conduct rat experiments for 2 to 4 weeks before sacrificing the animals. The reduction in the amount of low choline diet required and of difficultly prepared or expensive compounds related to choline chloride is important also.

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Male mice of the same strain and of approximately the same age and weight (15-25 g) are arranged in groups of 8-12 in the experiments described and kept on a standard diet until the day previous to the beginning of the experiment. During the experimental period the diet consists of powdered beef muscle (Difco), well extracted with alcohol and ether, 5.3%; powdered sucrose, 48%; beef fat, 38%; cod liver oil, 2%; agar, 2%; salt mixture (U.S.P. XI No. 2), 4%; yeast concentrate,† 0.75%. The chemical to be tested is dissolved in a small amount of water and very thoroughly incorporated with the food; fresh supplies of the latter are placed daily in beakers (50 cc) which are wired to the cages; the weight of food residues is recorded daily. The animals waste little of their food supply when fed 2 g per mouse per day. The change in weight during a 6-day period may vary between ±10% but usually shows a small gain. The animals are decapitated and the livers analyzed for "total fat," which consists of the weight of the residue from the petroleum ether soluble fraction of the pooled livers, following saponification and acidification.

The efficacy of choline chloride in preventing deposition of "fat" in the livers of the mice may be expressed as follows: $\frac{x-y}{x-3.5} \cdot 100 =$ percentage effectiveness of test dose; where x represents the percentage of "fat" in the livers of control animals; y, the percentage of "fat" in the livers of animals which received choline chloride and 3.5 the average percentage of "fat" in the livers of normal mice.

In Fig. 1 A the results of such calculations are plotted against

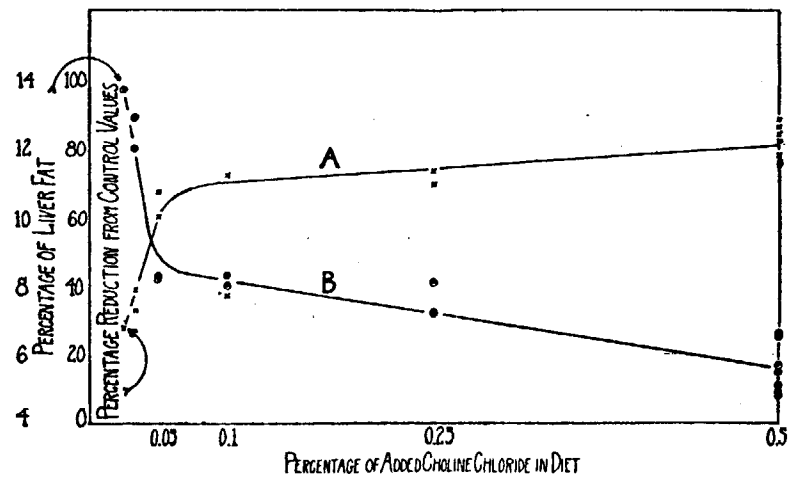


FIG. 1.

† Generously supplied by Anheuser-Busch, Inc.

dosage of choline chloride. In Fig. 1 B the actual percentage of liver fat in the test animals, without reference to the value in the controls, is plotted against choline chloride dosage.

The curves indicate that at the higher dosage levels the effect of increasing the concentration of choline chloride in the diet is slight and only at levels of less than 0.05%, under the conditions described, is there a marked correlation between concentration and action. It would appear evident that in assaying the activity of any lipotropic substance it is important to use relatively small doses in order to obtain the most significant relation between the size of the dose and the lipotropic action produced.

It will be appreciated that under any conditions the amount of "extra fat" actually "transported" or otherwise disposed of due to the influence of added choline chloride is relatively small. A 20 g mouse under the experimental conditions will deposit only about 0.15 ± 0.05 g of "extra fat" in the liver in the absence of added choline chloride, although consuming nearly 5 g of fat during the 6-day test period. From the figures it is evident that the smaller the dose of choline chloride the greater is its relative efficiency in preventing the deposition of a portion of this small amount of fat, that is, more fat is "transported" per molecule of choline chloride.

Summary. A 6-day method for the assay of lipotropic substances in the mouse is described. It is shown that a significant relation between the concentration of choline chloride added to a low choline-high fat diet and the lipotropic effect produced exists only at levels approaching the minimum effective dose, under the experimental conditions described.

Yamaguchi, F.: STUDIES ON THE EXPERIMENTAL ATHEROMATOUS ARTERIOSCLEROSIS. I. RELATION BETWEEN THE DEVELOPMENT OF ARTERIOSCLEROSIS AND LIPID PATTERN OF BLOOD AND LIVER IN THE CHOLESTEROL-FED COCKERELS. Taishitsu Igaku Kenkyusho Hokoku, Vol. 9, pp. 311-330, 1959.

The cause of arteriosclerosis has been pursued since late 19th century, but no conclusive explanation has yet been made. Attention has been directed to a number of factors such as neurological, psychological, hereditary, local, endocrine, dietary, metabolic, etc., but at the present, multiple factors are considered to be responsible for this condition. Most of all, atheromatous arteriosclerosis has been known to be deeply related to abnormality in lipid metabolism, but dietary factor also contributes to this condition. Rosenthal [1] recognized lower incidence of atheromatous arteriosclerosis among the Japanese, Chinese and natives of Okinawa whose fat intake is low as compared to that of the Americans and Europeans. Alexander [2] attributed the low incidence of the sclerosis of the aorta and coronary artery among the natives of South Africa to their low fat intake, and observed a higher incidence among the natives who are accustomed to the diet the whites are accustomed to. Many investigators have acknowledged the correlation among various factors associated with arteriosclerosis such as high-fat diet, increased lipids in the blood, and the deposition of fat on the vascular walls. For instance, Windaus [3], Lande and Sperry [4], and Page [5] stated that the severity of sclerosis of the aorta is proportional to the blood cholesterol level, and Smith [6] and Duff [7] demonstrated a positive relationship between injuries on vascular walls and the accumulation of cholesterol and cholesterol ester in their experiments using rabbits. Schoenheimer [8] reported that the cholesterol level in sclerotic aorta was higher than normal aorta. There have been a large number of papers discussing high incidences of cholesterinosis among individuals with angina pectoris and coronary arteriosclerosis [6, 9-13]. Morrison [14] stat4d that the cholesterol level in the left anterior descending coronary artery of a patient who died of acute arteriosclerosis was approximately 4 times higher than the control. Recently, the research in this area has been expanded to include the investigation of the abnormal decomposition of the substances related to the cholesterol level. In relation to abnormal lipid metabolism, the fractions of lipids and lipoids are being scrutinized. Lipids and lipoids are contained in blood plasma in the form of lipoprotein molecule. Measuring the lipoprotein level by Cohn's method, Barr [15] and Russ [17] observed not only an increase in blood cholesterol but a decrease in the cholesterol in α-lipoprotein fraction and an increase of the cholesterol in β-lipoprotein fraction in the serum. Furthermore, Gofmann [16] demonstrated, by the supercentrifugal sedimentation method, that the Sf_{10-30} fraction exhibited a close relationship with arteriosclerosis and, when the coronary arteriosclerosis was used as the standard for the judgment of clinical arteriosclerosis, the Sf_{10-20} and Sf_{35-100} fractions showed a correlation to the condition. Changes in these substances influence the solubility and precipitation of lipids, and subsequently cause the fat to be deposited on arterial walls. However, it is still problematic to relate arteriosclerosis to a specific factor, although it must be noted that lipids play an important role in the pathogenesis of this disease.

With a growing population of the aged in recent years, the diseases derived from arteriosclerosis have shown an alarming rate of increase.

Thus, the prevention and treatment of this disease has become a major assignment of the medical field. In the illness in which the age is also an influential factor, the elimination of the condition or cause of the hardening of arteries is enevitably an important preventive as well as therapeutic measure, but, in view of the fact that the individuals with this illness have abnormal lipid metabolism, i.e., cholesterinosis and hyperlipidemia, it is naturally important to pay attention to the amount of fat and cholesterol contained in their diet. In such an attempt, a low-fat diet should be prescribed in consideration of the abnormal lipid metabolism. According to Oshima's report [67], however, the serum cholesterol level in animal is directly influenced by the cholesterol content of the feed, but the fat intake is more influential on human cholesterol level than the cholesterol content in the diet. In other words, he demonstrated that the serum cholesterol level in animal could be reduced by lower intake of holesterol whereas a change in cholesterol content in human diet had minimal influence on the serum cholesterol level. He ascribed this observation to the fact that a human body contained endogenic cholesterol in an amount far greater than that of exogenic cholesterol. The production of cholesterol in vivo is increased by excessive intake of fat or even carbohydrate, and this is assumed to be the reason for the effect of total caloric or fat intake on the serum cholesterol level. Needless to say, excessive intake of fat or carbohydrate or high-calorie diet must be avoided. Other measures should also be formulated taking the effects of the increased production of endogenous cholesterol and lipoprotein into consideration. Furthermore, the use of anticholesterol and lipotropic agents which are considered to control hypercholesterolemia and hyperlipidemia and improve lipid metabolism, may prove effective and achieve alleviation and control of arteriosclerosis. Since the pathogenic mechanism of this disease is extremely complex and also involves the effect of aging, the effect and significance of the drug therapy naturally require full investigation, and valid standards for the judgment of their therapeutic effect. With regard to this analysis, the author studied the effects of several drugs on the lipid pattern of the blood and liver, and their inhibitory action on the pathogenesis of arteriosclerosis. The drugs employed in this experiment were lipotropic agents of methionine, choline and inositol, chondroitin sulfate, a type of acidic mucopolysaccharide considered to be an intercellular substance, and vitamin B_{12} which is assumed to possess lipotropic action.

EXPERIMENTAL PROCEDURE AND MATERIALS

As in the previous experiment, approximately 3 months old cockerels (White Leghorns) weighing 1.0 to 1.2 kg were used. Each group consisted of 15. The feed was prepared by mixing 1 g/kg of body weight of cholesterine (U.S.P., XIV) and 5 cc of cottonseed oil to the basal feed, as in the preparation of the feed given to the cholesterol group in the previous experiment. After each feeding of the above ration, the birds were given a small amount of greens mixed to the basal feed, the type of feed given to the control group in the previous experiment, to supplement the feed intake. Water was given upon demand. The drug was mixed in the feed with cottonseed oil and cholesterol for oral administration, or injected by the intramuscular route once daily. The dose was adjusted weekly in correspondence to the change in body weight. As discussed in the previous report, the blood and liver lipid levels were determined by the method employed by Katura, Hatayama, and Tamiya [15]. The amount of lipids in the blood was measured before the treatment and at 2-week intervals for a period of

16 weeks. As a rule, five from each group were killed by bloodletting each month during the experiment for microscopic examination of the sclerotic alteration in aortic arch, thoracic aorta, and abdominal aorta. The degree of sclerotic alteration was determined according to the system developed by Hakamada [19]. In autopsy, the liver and the heart were weighed, and the fat in the liver was measured immediately after the withdrawal of the blood. Methionine was administered at 1 g per kg of body weight for 8 weeks, but the dosage was reduced to 0.5 g per kg of body weight thereafter due to loss of weight.

TABLE 1. MODE OF ADMINISTRATION

a 群	i コレステリン*	植油	k 薬剤処置量**	p 投与法
b メチオニン群	1gr	5cc	l dlメチオニンを7週目まで1gr, 以後0.5gr	q 経口
c コリン群	〃	〃	m 塩化コリン 0.25gr	q 経口
d イノシット群	〃	〃	n イノシット 10mg	r 筋注
e コンドロイチン硫酸群	〃	〃	o コンドロイチン硫酸 5mg	r 筋注
f Vitamine B_{12} 群	〃	〃	Vitamine B_{12} 15r	r 筋注
g 「コ」 酸 群	〃	〃	0	
h 対照群	0	0	0	

* Crystalline, U.S.P. XIV.
** Daily dose per kg of body weight.

Keys: a, group; b, methionine group; c, choline group; d, inositol group; e, chondroitin sulfate group; f, vitamin B_{12} group; g, cholesterol group; h, control group; i, cholesterine; i, cottonseed oil; k, dosage of drug; l, dl methionine at 1g up to the 7th week, 0.5 g thereafter; m, choline chloride; n, inositol; o, chondroitin sulfate; p, mode of administration; q, oral; r, intramuscular.

EXPERIMENTAL RESULTS

1. EFFECTS ON BODY WEIGHT AND LIPID PATTERN OF BLOOD

In the previous study, the author compared the lipid pattern in the blood and liver of the control group and cholesterol group, and the critical limit of the fat fraction in the blood was obtained using 180 three-month old cockerels. With the critical limit as the normal range, the changes in blood lipid level due to the drug treatment were examined with the cholesterol group as the control.

The changes in the blood lipid fraction and the body weight of the groups treated with methionine, choline, inositol, chondroitin sulfate, and vitamin B_{12} are shown in Tables 2 - 6.

A. BODY WEIGHT

Changes in body weight are plotted in Figure 1.

(a) Methionine group: At 1 g per kg of body weight of methionine, this group showed an upward tendency as the cholesterol group for the first 4 weeks, but a slight decrease occurred in the 6th week and a sharp decrease in the 8th week. For this reason, the dosage was reduced to 0.5 g per

TABLE 2. CHANGES IN BLOOD LIPID PATTERN (mg/dl) AND BODY WEIGHT (g) DUE TO METHIONINE

	a 処置前	b 処置後2週	4 週c	6 週c	8 週c	10 週c	12 週c	14 週c	16 週c
羽数 d	14	14	14	14	14	10	10	5	5
平均体重 e / 脂肪分画 f	1139	1250	1407	1385	1296	1540	1801	1932	2308
燐脂 g	169.0	159.0	168.8	194.5	156.2	113.4	175.0	133.4	184.6
総コレステリン h	119.0	200.9	211.2	126.5	213.1	266.2	230.4	136.6	185.6
遊離コレステリン i	81.6	164.0	162.2	76.5	172.7	215.2	178.5	98.0	153.4
エステルコレステリン j	67.4	96.9	49.0	50.0	40.4	51.0	51.9	38.6	32.2
中性脂肪 k	202.4	605.3	534.1	567.2	450.7	463.3	448.6	582.8	505.4
脂肪酸 l	413.0	901.4	458.5	578.4	573.1	553.1	654.9	539.4	561.6
総脂 m	562.0	1026.2	944.4	919.2	845.0	804.5	877.2	876.8	895.6
C / P	0.88	1.07	1.34	0.65	1.36	1.86	1.31	1.02	1.01
E / T	0.46	0.48	0.23	0.40	0.19	0.19	0.23	0.28	0.17

TABLE 3. CHANGES IN BLOOD LIPID PATTERN (mg/dl) AND BODY WEIGHT (g) DUE TO CHOLINE CHLORIDE

	a 処置前	b 処置後2週	4 週c	6 週c	8 週	10 週	12 週	14 週	16 週
羽数 d	15	15	15	15	15	10	10	5	5
平均体重 e / 脂肪分画 f	1157	1413	1725	1900	2050	2232	2298	2300	2306
燐脂 g	181.8	166.1	186.8	210.2	176.0	156.2	188.2	194.5	203.9
総コレステリン h	197.6	210.2	179.6	191.8	261.2	191.8	247.4	210.1	268.3
遊離コレステリン i	98.0	71.4	132.6	130.6	140.7	66.3	93.4	106.1	185.6
エステルコレステリン j	99.6	138.8	47.0	61.2	120.5	125.5	154.0	104.0	82.7
中性脂肪 k	218.2	355.6	670.5	728.6	704.8	741.0	683.5	416.5	314.0
脂肪酸 l	441.8	466.2	428.5	517.2	523.0	556.5	532.8	470.6	683.8
総脂 m	659.8	833.8	1066.0	1168.2	1208.5	1166.8	1214.6	885.6	837.5
C / P	1.09	1.26	0.96	1.9	1.48	1.23	1.32	1.08	1.32
E / T	0.50	0.64	0.26	0.32	0.46	0.53	0.62	0.50	0.31

TABLE 4. CHANGES IN BLOOD LIPID PATTERN (mg/dl) AND BODY WEIGHT (g) DUE TO INOSITOL

	処置前a	処置後2週b	4 週c	6 週	8 週	10 週	12 週	14 週	16 週
羽数 d	14	14	14	14	14	10	10	5	5
平均体重 e / 脂肪分画 f	1051	1267	1417	1521	1586	1849	1926	2040	1930
燐脂 g	186.0	221.4	150.7	160.6	177.8	152.8	167.6	201.5	269.8
総コレステリン h	150.9	140.8	381.4	293.8	323.2	297.2	309.1	259.1	279.4
遊離コレステリン i	123.4	42.8	191.7	83.6	161.5	120.3	130.6	110.8	151.0
エステルコレステリン j	27.5	98.0	189.7	210.2	161.7	170.9	178.5	118.3	128.4
中性脂肪 k	270.3	522.6	429.6	366.6	390.8	436.7	357.7	396.9	380.2
脂肪酸 l	367.4	568.4	555.0	579.4	572.8	593.4	593.9	644.9	525.0
総脂 m	624.3	948.6	955.1	1121.0	992.0	992.4	941.4	933.8	1009.0
C / P	0.81	0.63	2.53	1.83	1.82	1.95	1.84	1.27	1.04
E / T	0.18	0.70	0.50	0.72	0.50	0.58	0.57	0.46	0.46

Keys: a, before treatment; b, 2 weeks after treatment; c, weeks; d, number of birds; e, average body weight; f, fat fraction; g, phospholipid; h, total cholesterol; i, free cholesterol; j, cholesterol ester; k, neutral fat; l, fatty acid; m, total lipid

TABLE 5. CHANGES IN BLOOD LIPID PATTERN (mg/dl) AND BODY WEIGHT (g) DUE TO CHONDROITIN SULFATE

	処置前[a]	処置後2週	4 週[c]	6 週	8 週	10 週	12 週	14 週	16 週[c]
羽 数[d]	12	12	12	12	12	8	8	4	4
平均体重[e] / 脂肪分画[f]	1073	1176	1333	1491	1631	1807	1912	1838	1845
燐脂質[g]	123.5	142.0	150.6	267.0	221.3	259.9	220.2	207.3	255.6
総コレステリン[h]	188.7	265.2	165.2	233.6	279.5	287.6	274.4	490.6	432.5
遊離コレステリン[i]	127.5	177.4	83.6	128.5	147.3	158.1	142.8	304.0	255.0
エステルコレステリン[j]	66.2	87.8	81.6	105.1	132.2	129.5	131.6	186.6	177.5
中性脂肪[k]	321.3	496.2	421.9	282.0	304.7	396.2	386.3	388.6	346.8
脂肪酸[l]	363.0	555.0	646.0	305.3	426.2	428.5	580.5	677.1	714.8
総脂質[m]	671.4	957.8	791.3	867.3	887.2	1024.0	961.2	1201.2	1145.0
C / P	1.53	1.87	1.01	0.88	1.26	1.11	1.25	2.38	1.69
E / T	0.35	0.33	0.53	0.45	0.49	0.46	0.48	0.38	0.41

TABLE 6. CHANGES IN BLOOD LIPID PATTERN (mg/dl) AND BODY WEIGHT (g) DUE TO VITAMIN B_{12}.

	処置前[a]	処置後2週	4 週[c]	6 週[c]	8 週[c]	10 週[c]	12 週[c]	14 週[c]	16 週[c]
羽 数[d]	11	11	11	11	11	8	8	4	4
平均体重[e] / 脂肪分画[f]	1138	1322	1418	1552	1607	1670	1733	1825	2138
燐脂質[g]	197.4	153.4	205.9	345.6	350.7	387.7	355.0	318.1	303.9
総コレステリン[h]	207.1	361.2	394.7	291.7	440.6	420.2	387.6	444.7	426.4
遊離コレステリン[i]	156.1	182.6	302.9	207.1	200.0	211.1	179.5	173.4	219.3
エステルコレステリン[j]	51.0	184.6	91.8	84.6	240.6	209.1	208.1	271.3	207.1
中性脂肪[k]	217.8	366.5	402.5	450.7	369.9	337.8	441.6	414.8	483.6
脂肪酸[l]	296.4	717.1	529.5	431.8	912.4	697.1	580.5	621.6	644.9
総脂質[m]	683.9	1001.6	1060.0	1140.5	1310.4	1325.3	1351.2	1345.8	1345.3
C / P	1.05	2.36	1.92	0.84	1.26	1.08	1.09	1.43	1.40
E / T	0.23	0.51	0.23	0.29	0.55	0.50	0.54	0.61	0.49

Keys: a, before treatment; b, 2 weeks after treatment; c, weeks; d, number of birds; e, average body weight; f, fat fraction; g, phospholipid; h, total cholesterol; i, free cholesterol; j, cholesterol ester; k, neutral fat; l, fatty acid; m, total lipid

kg of body weight, as a result of which a sudden increase in body weight occurred, reaching the control level in the 16th week.
(b) Choline group: Sudden increases were noted in the 2nd, 4th, and 6th weeks, and the body weight reached 2200 g in the 10th week, and 2300 g in the 12th week. No further increase was noted thereafter. This group attained the same level as the cholesterol group in the 16th week, with a remarkably high rate of increase up to that level.
(c) Inositol group: This group exhibited the same pattern of increase as the cholesterol group except for a slight drop in the 16th week.
(d) Chondroitin sulfate group: The pattern up to the 12th week resembled that of the cholesterol group, but decreases were shown between the 14th and the 16th weeks. As compared to 2262 g of the cholesterol group in the 16th week, this group gave 1845 g, a significantly lower value.
(e) Vitamin B_{12} group: The pattern up to the 8th week was similar to that of the cholesterol group, but the increase from the 10th to the 14th week was moderate. A sudden increase occurred in the 16th week. In general, with the exception of methionine group with a sharp decrease at a time, these groups followed the pattern shown by the control. The choline group

indicated extremely favorable growth, with values surpassing those of the control and cholesterol groups, but other four groups showed slightly inhibited pattern of growth as compared to that of the cholesterol group after the 12th week.

B. BLOOD LIPID PATTERN

(1) Phospholipid: Changes in phospholipid are illustrated in Figure 2. Methionine Group: Fluctuations within a range of 140 - 180 mg/dl were noted throughout the experimental period. The group did not show a sharp increase as shown by the cholesterol group. Choline group: The fluctuations remained within a normal range throughout the period. Inositol group: Changes were within normal range for the first 14 weeks, but a slight increase above the upper limit of normal range was noted in the 16th week. Chondroitin sulfate group: Elevation was moderate, slightly reaching beyond the upper limit of normal range in the 6th, 10th, and 16th weeks. As compared to the cholesterol group, the increases were extremely slight. Vitamin B_{12} group: This group maintained the normal range of fluctuations for the first 4 weeks, but a sudden rise occurred from the 6th week, reaching a peak in the 19th week. A slight drop was noted thereafter, but the phospholipid level as of the 16th week was high, 300 mg/dl. The increases were generally more notable than those in other 4 groups, but were still slight as compared to those of the cholesterol group.

Summarizing the above results, the phospholipid levels of the methionine, choline, and inositol groups remained within normal range, and the production of phospholipid was more or less totally suppressed. Although the suppressive effect of chondroitin sulfate is slightly weaker than that of three other drugs, it still checked the elevation of phospholipid level. Vitamin B_{12} group exhibited higher than normal level after 6 weeks, with intermediate values between that of the cholesterol group and normal value.

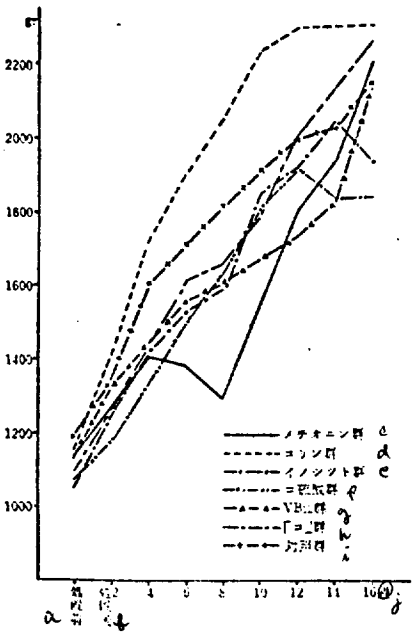


Figure 1. Changes in Body Weight.

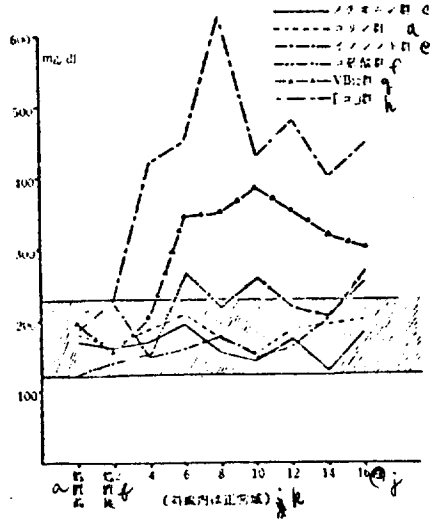


Figure 2. Changes in Phospholipid

Keys: a, before treatment; b, after treatment; c, methionine group; d, choline group; e, inositol group; f, chondroitin sulfate group; g, V B_{12} group; h, cholesterol group; i, control group; j, weeks; k, (the shaded area is the normal range).

(2) Total cholesterol. The changes in total cholesterol are plotted in Figure 3. Methionine group: Generally, the total cholesterol level remained within normal range throughout the period except for a slight rise in the 10th week. Choline group: Same as the methionine group. Inositol group: Fluctuations within normal range were observed up to the 2nd week, a moderate increase in the 4th week, and a slight drop followed by a slight increase thereafter. The increases were slight, as compared to those shown by the cholesterol group, but the values were generally elevated above the control throughout the period. Chondroitin sulfate group: A rise slightly above normal range was observed in the 14th week, but sharp increases followed thereafter, with values close to those of the cholesterol group. Vitamin B_{12} group: A gradual rise took place from the 2nd week, although the rate of increase was by far lower than that of the cholesterol group. No sign of suppression in total cholesterol was noted, as compared to the cholesterol group.

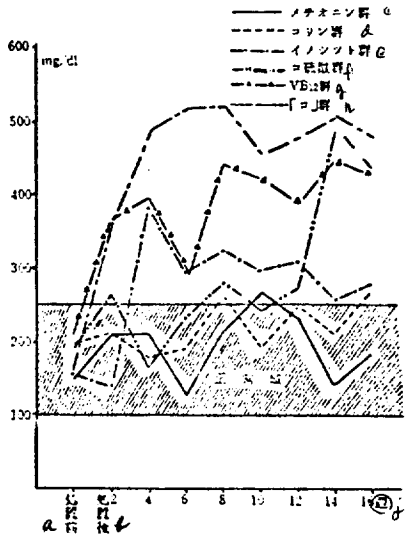


Figure 3. Changes in Total Cholesterol. (Koeys (Keys, same as Figures 1 & 2).

Summarizing the above results, the methionine and choline groups showed no elevation of total cholesterol level, and the inositol group, an intermediate degree of increase in the 4th week and a gradual decrease thereafter. The final level of the inositol group exceeded the upper limit of the normal range, but the rate of increase was far lower than that of the cholesterol group. Chondroitin sulfate group exhibited a sudden rise from the 14th week, to a value close to that of the cholesterol group. Vitamin B_{12} group gave a value exceeding that of the cholesterol group in the 2nd week, and its pattern of total cholesterol resembled that of the cholesterol group with notable increases.

(3) Free cholesterol. The changes in free cholesterol level are plotted in Figure 4. Methionine group: Except for a slight rise in the 10th week, the values remained within normal range. Choline group: Fluctuations within normal range were noted throughout the period. Inositol group: Changes remained within normal range at all times. Chondroitin sulfate group: The values up to the 12th week were within normal range, but those after the 14th week reached close to those of the hholesterol group. Vitamin B_{12} group: A sudden rise occurred in the 4th week, to 302.9 mg/dl, but a downward tendency continued after 6 weeks, lowering the level near the upper limit of normal range by the 16th week.

In summary, no increase in free cholesterol was shown by the methionine, choline, and inositol groups, with changes generally remaining within normal range. The chondroitin sulfate group maintained normal level up to the 12th week, but showed a sharp increase, to the level of the cholesterol group, after 14 weeks. Vitamin B_{12} group exhibited a pattern of increase similar to that of the cholesterol group up to the 4th week, and a downward trend thereafter, to a level close to the upper limit of normal range.

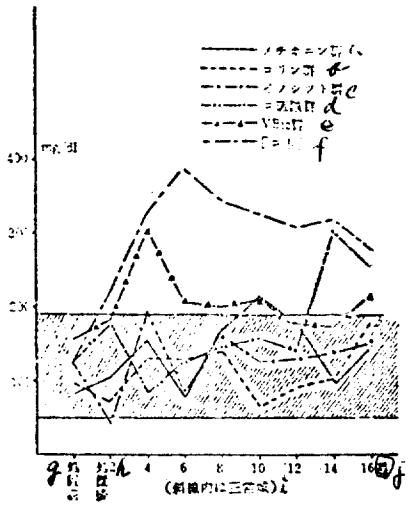


Figure 4. Changes in Free Cholesterol

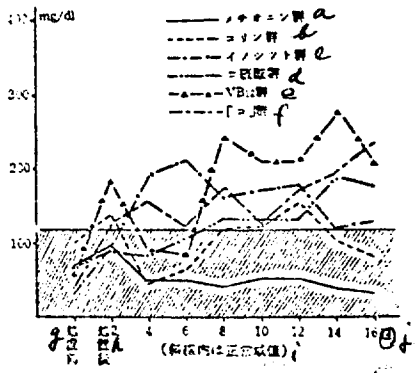


Figure 5. Changes in Cholesterol Ester

Keys: a, methionine group; b, choline group; c, inositol group; d, chondroitin sulfate group; e, vitamin B_{12} group; f, cholesterole group; g, before treatment; h, after treatment; i, (the shaded area is the normal range); j, weeks

(4) Cholesterol Ester. The cholesterol ester levels of all groups are compared in Figure 5. Methionine group: Values remained within normal range at all times. Choline group: The value reached slightly above normal range between the 8th and 12th weeks, but remained normal before and after that period. Inositol group: An intermediate degree of increase was noted between the 4th and 12th weeks, but the value began to drop thereafter, with the final value slightly higher than the upper limit of the normal range. Chondroitin sulfate group: Changes up to the 6th week remained within normal range, but a moderate rise continued, to 177.5 mg in the 16th week. Vitamin B_{12} group: Marked variation was noted, with a value equal to that of the cholesterol group in the 2nd week. The value dropped temporarily in the 4th and 6th weeks, but a sharp increase occurrred after 8 weeks, to a level beyond that of the cholesterol group.

In summary, the methionine group showed no increase in cholesterol ester, the choline group a slight temporary increase between the 8th and the 12th weeks, and inositol group, sharp increases comparable to those of the cholesterol group in the 4th and 6th weeks, a constant level in the 8th, 10th, and 12th weeks, and a gradual decrease thereafter as compared to a continuous rise of the cholesterol group, inositol group indicated gradual decrease. The values shown by the inositol group was above normal range throughout the experimental period. Chondroitin sulfate group exhibited an upward trend from the 8th week, reaching the level of the cholesterol group in the 14th to 16th weeks. Vitamin B_{12} group indicated sharp increases for 8 weeks, with values close to those of the cholesterol group.

(5) Neutral fat. The fluctuations of the neutral fat level are illustrated in Figure 6. Methionine group: Changes were considerable. The neutral fat level in the 2nd week exceeded that of the cholesterol group, with a value of 606 mg/dl, and values over 450 mg/dl thereafter. Choline group:

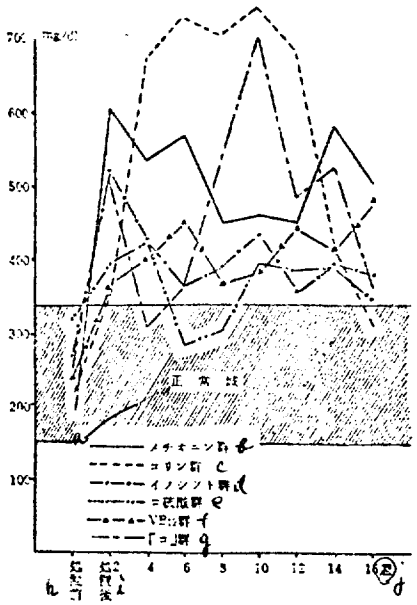
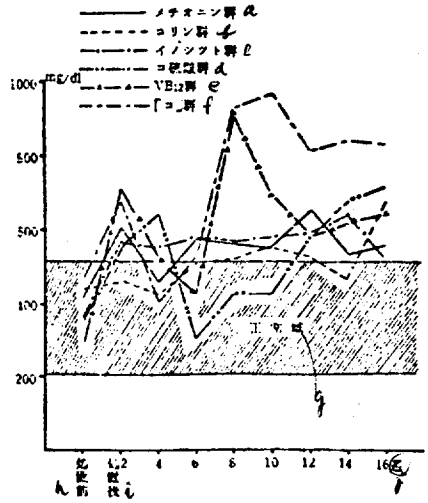


Figure 6. Changes in Neutral Fat
Keys: a,normal range; b, methionine group; c, choline group; d, inositol group; e, chondroitin sulfate group; f, V B_{12} group; g, cholesterol group; h, before treatment; i, after treatment; j, weeks

Considerable variations were shown. The values between the 4th and 12th weeks were high, with a peak of 741.0 mg/dl. However, a sudden drop occurred between the 14th and 16th weeks, to almost normal. The neutral fat level during the 4th - 12th week period was appreciably higher than that of the cholesterol group. Inositol group: The neutral fat level fluctuated beyond normal range, but the degree of increase was lower than that of the cholesterol group. Chondroitin sulfate group: The value exceeded 400 mg/dl in the 2nd to 4th weeks, but remained in the vicinity of 400 mg/dl thereafter. The degree of elevation was lower than that of cholesterol group except for the 2nd to 4th weeks as that of inositol group. Vitamin B_{12} group: The value exceeded normal range at all times, generally averaging approximately 400 mg/dl. The degree of elevation was slightly lower than that of the cholesterol group.

All groups indicated increases beyond normal range. The methionine group surpassed the cholesterol group in the 2nd to 6th weeks, and so did the choline group. Inositol group, chondroitin sulfate group and V. B_{12} groups also showed upward tendencies although the degree of increase was lower than that of the cholesterol group.



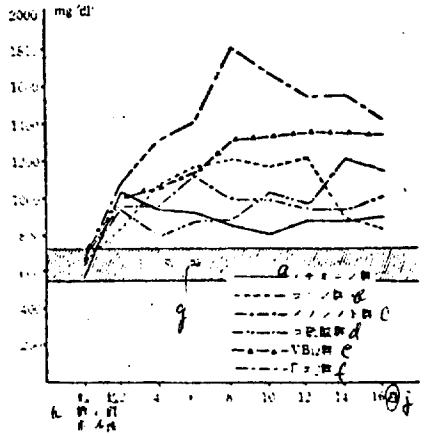


Figure 7. Changes in Fatty Acid. Figure 8. Changes in Total Lipid.
Keys: a, methionine group; b, choline group; c, inositol group; d, chondroitin group; e, Vitamin B_{12} group; f, cholesterol group; g, normal range; h, before treatment; i, after treatment; j, weeks.

(6) Fatty Acid. Changes in fatty acid level are plotted in Figure 7. Methionine group: An increase occurred already in the 2nd week, and the values after the 6th week remained near the upper limit of normal range, between 654.9 and 553.1 mg/dl. Choline group: The values between the 6th and the 14th weeks remained near the upper limit of normal range, but a sudden increaseoccurrred in the 16th week. Inositol group: The value increased slightly after 2 weeks, to approximately 570 mg/dl. Chondroitin sulfate group: Considerable variations were noted. A sudden, temporary rise occurred in the 2nd to 4th weeks, reaching 555.0 mg/dl, but the value returned to normal between the 6th and 10th weeks. A sharp increase occurred again in the 12th week. Vitamin B_{12} group: The value before the treatment was considerably low, 296.4 mg/dl, but it sharply rose to 717.1 mg/dl in two weeks. A temporary drop was noted in the 6th week, but a peak of 912.4 mg/dl was reached in the 8th week. A downward trend continued thereafter, with values of 621.6 and 644.9 mg/dl in the 14th and 16th weeks, respectively.

In summary, all groups exhibited an upward tendency. The elevation shown by the methionine, choline and inositol group was slight. The chondroitin sulfate group showed a temporary rise between the 2nd and the 4th weeks, but the value subsequently returned to normal. A sudden increase occurred after 12 weeks, although the rate of increase was still moderate as compared to that of the cholesterol group. Vitamin B_{12} group indicated increases comparable to those of the cholesterol group, reaching a peak of 912.4 mg/dl in 8 weeks, but this group subsequently showed a drop whereas the cholesterol group maintained a high level, over 900 mg/dl, through the end of the experiment.

(7) Total lipid. The total lipid levels of the drug-treated groups are plotted in Figure 8. Methionine group: A slightly higher level was maintained after the 2nd week. Choline group: A moderately elevated pattern was shown between the 2nd and the 12th weeks, and a slightly high level after the 14th week. Inositol group: An intermediate degree of elevation continued from the 2nd week. Chondroitin sulfate group: It began to exhibit an upward trend from the 2nd week, with a degree of elevation rising with time and a value of approximately 1200 mg/dl after the 14th week. Vitamin B_{12} group: An upward tendency was maintained from the 2nd week, with a value above 1300 mg/dl after the 8th week. However, the value is still low as compared to that of the cholesterol group.

Summarizing the above results, all the drug-treated groups exhibited elevation in total fatty acid level, the degree of elevation being minimal in theorder of methionine group and inositol group. Choline group maintained values within a range of 1000 to 1200 mg/dl except for the 14th and 16th weeks when the value dropped to 800 mg/dl, the degree of elevation being generally to an intermediate degree. Chondroitin sulfate group showed a gradual rise to 1200 mg/dl from 800 mg/dl, the increase after the 8th week being particularly notable. Vitamin B_{12} group exhibited the most notable elevation among the drug-treated groups, which, however, was still minimal as compared to that shown by cholesterol group.

(8) C/P (total cholesterol/phospholipid). In a healthy animal, the C/P ratio remains within a specific range, but arteriosclerosis is said to increase the ratio. The fluctuations in blood C/P following the treatment are illustrated in Figure 9. As stated in the previous report, the cholesterol group showed values higher than those of the control,

distributed primarily within a range of 0.8 to 1.2. The distribution of C/P ratio was examined by dividing the values into four grades: under 0.8, 0.8 - 1.2, 1.2 - 1.4, and above 1.4. The methionine group gave a distribution of under 0.8 (1), 0.8 - 1.2 (3), 1.2 - 1.4 (3), and above 1.4 (1), an average value being 1.20. The choline group showed a distribution of 0.8 - 1.2 (3), 1.2 - 1.4 (4), and above 1.4 (1), with an average of 1.22. The inositol group showed a distribution of under 0.8 (1), 0.8 - 1.2 (1), 1.2 - 1.4 (1), and above 1.4 (5), with an average of 1.61. The chondroitin sulfate group showed a distribution of 0.8 - 1.2 (3), 1.2 - 1.4 (2), and over 1.4 (3), with an average of 1.44. The vitamin B_{12} group exhibited a distribution of 0.8 - 1.2 (3), 1.2 - 1.4 (1), and above 1.4 (4), with an average of 1.42. As shown above, the C/P ratio failed to exhibit a specific tendency and varied over a wide range. Comparing the groups in terms of average C/P, the drug-treated groups obviously gave higher value than the control and cholesterol groups. The average values of the methionine group and choline group were generally low, 1.20 and 1.22, respectively. The inositol group exhibited variations over a broad range, and the highest average, 1.61. The chondroitin and Vitamin B_{12} group also exhibited wide ranges of distribution, but their average values were 1.44 and 1.42, respectively, between those of the methionine and choline groups and that of the inositol group.

(9) E/T (cholesterol ester/total cholesterol). The E/T ratio of a healthy human is said to be within a range of 0.6 to 0.8. In the case of animals, each species shows a specific value. Changes in E/T following the drug treatment are illustrated in Figure 10. The E/T of the control and cholesterol groups varied within a range of 0.2 - 0.5, For comparison purpose, the rates were graded into 4 steps: under 0.20, 0.2 - 0.3, 0.3 - 0.5, and above 0.5. The methionine group gave an average of 0.43 with a distribution of under 0.2 (3), 0.2 - 0.3 (3), and 0.3 - 0.5 (3). The inositol group had an average of 0.559, with the distribution of 0.3 - 0.5 (3) and above 0.5 (5). The chondroitin sulfate group showed a distribution of 0.3 - 0.5 (7) and over 0.5 (1) with an average of 0.44. The vitamin B_{12} group included two 0.2 - 0.3 and two 0.3 - 0.5, and 4 over 0.5, with an average of 0.46.

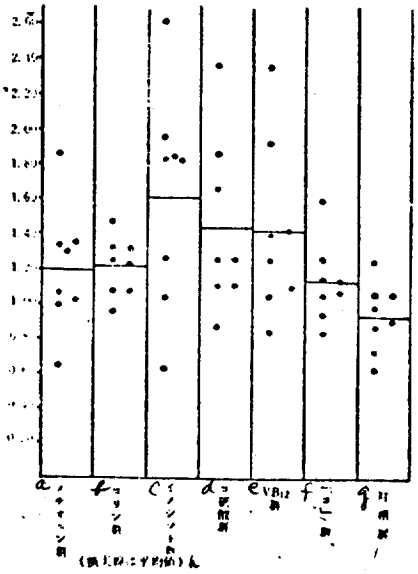


Figure 9. C/P Distribution

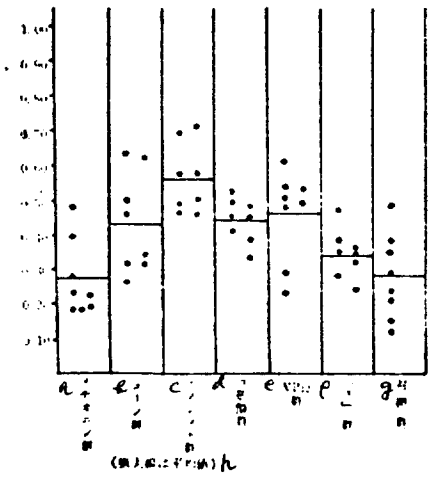


Figure 10. E/T Distribution

Keys: a, methionine group; b, choline group; c, inositol group; d, chondroitin sulfate group; e, V B_{12} group; f, cholesterol group; g, control group; h, (horizontal line is the average).

Since the control group showed a distribution of below 0.2 (2), 0.2 - 0.3 (2), 0.3 - 0.5 (3), and over 0.5 (0), with an average of 0.28, and the cholesterol group, a distribution of 0 for below 0.2, 2 for 0.2 - 0.3, 6 for 0.3 - 0.5, and 0 for over 0.5, with an average of 0.34, the distribution and the average of the methionine group were closest to those of the control, and the values are lower than those of the cholesterol group. The distribution exhibited by the choline group is characterized by a wide range of variation, generally with greater distribution of higher values, and its average value is higher than that of the control or the cholesterol group. Many belonging to the inositol group gave high values, thus, the group's average value being highest among the drug-treated groups. The high E/T value may be attributed to an increase in cholesterol ester. As compared to the cholesterol and control groups, the chondroitin sulfate group gave high values in larger number, resulting in a higher average value, 0.44. Vitamin B_{12} group gave relatively low values in the 4th and 6th weeks, but values thereafter were considerably high, elevating the average value to 0.46, which, however, is still lower than that of the inositol group.

II. EFFECTS ON THE BODY WEIGHT AND THE WEIGHTS OF THE LIVER AND HEART.

The cockerels were weighed in the 2nd, 3rd, and 4th months, and the measured values are given in Table 7 along with the weights of the liver and heart. Table 8 provides the same data for the cholesterol and control groups.

The administration of methionine at 1 g/kg of body weight resulted in loss of body weight. It also caused reduction in the weight of the organs in the 2nd month, to 33.6 g for the liver and 7.1 g for the heart, considerably lower than those of the control group. However, the reduction of dosage to 0.5 g/kg of body weight achieved a sharp increase in weight to above the control level in the 3rd and 4th months, and above the level of the cholesterol group in the 4th month. The choline group began to show an increase in body weight from the 2nd month, which contributed to the heart and liver heavier than those of other groups. The weights were comparable to those of the cholesterol group in the 3rd and 4th months, and the weight of the heart exceeded the latter from the second month. The inositol group, which indicated suppressed growth of body weight showed the weight of the liver between that of the control and that of the cholesterol group, although its average weight of the heart exceeded that of the cholesterol group after the 3rd month. The chondroitin sulfate group exhibited no significant increase in liver weight, and its average heart weight was below that of the control group. Table 9 shows the heart/body weight ratios and liver/body weight rations of all the groups. The experimental groups generally gave larger values than that of the control group, but the rate became smaller with time. The cholesterol group gave a rage of below 0.033 in the 2nd month, but suddenly became smaller in the 3rd and 4th months, but other 4 groups, excluding the chondroitin sulfate group, gave larger values than that of the cholesterol group in the 4th month.

The heart/body weight ratio of the control and cholesterol groups reached peaks in the 2nd months, but subsequently became smaller. In the experimental groups, the rate was larger in the 4th month than in the 2nd month. The heart/body weight ratio of the methionine group was below that of the control group, but exceeded the latter from the 3rd month, reaching the level of the cholesterol group in the 4th month. Vitamin B_{12} group

ranked between the control and the cholesterol group, but other 3 groups gave lower values than that of the cholesterol up to the 2nd month and surpassed the latter in the 3rd and 4th months. These data suggest that the drug-treated groups has the heart relatively large for the body.

Summarizing the above results, the liver and heart of the experimental groups gained wieght at a higher rate than the body itself.

TABLE 7. CHANGES IN BODY WEIGHT, AND THE WEIGHT OF THE LIVER AND THE HEART.

	a メチオニン群 (g)				b コリン群 (g)				c イノシット群 (g)			
	No	d体重	e肝重量	f心臓重量	No	d体重	e肝重量	f心臓重量	No	d体重	e肝重量	f心臓重量
h二ヶ月	C 6	1300	34.8	8.7	D 1	1830	43.0	14.7	E 2	1500	42.5	10.4
	2	1490	37.6	6.3	2	2300	64.0	19.1	3	2040	51.5	15.5
	12	1300	38.4	6.8	3	1900	42.7	13.8	10	1440	36.4	8.0
	3	1230	23.6	6.4	4	2120	54.3	16.5	14	1790	46.5	11.6
					6	1970	39.4	13.1				45.5
	g平均	1330	33.6	7.1	g平均	2024	48.6	15.4	g平均	1692.5	44.2	11.4
i三ヶ月	C14	2170	49.0	11.5	D 7	2100	55.5	18.5	E 1	2220	42.0	15.7
	15	1800	38.5	10.5	9	2400	51.5	16.0	5	1900	40.5	13.8
	4	1700	45.0	10.5	10	1150	55.0	18.2	12	2000	40.5	13.7
	11	1880	46.5	11.3	11	2400	50.2	20.0	13	1920	38.7	13.7
	10	2000	45.0	12.8	12	2470	49.0	20.5	15	1940	43.7	15.2
	g平均	1910	44.8	11.3	g平均	2301	52.2	18.6	g平均	1996	41.	14.4
j四ヶ月	C 5	2160	56.7	14.7	D 5	2250	57.5	22.3	E 4	1800	51.7	14.5
	7	1650	39.5	10.0	8	2560	68.0	20.0	7	2180	50.0	20.5
	8	2800	67.7	20.2	12	2320	47.7	20.0	8	1800	41.8	20.0
	9	2140	49.0	10.7	14	2340	58.5	11.7	9	1950	47.2	14.7
	13	2300	60.8	15.5	15	2320	51.0	19.5	11	1880	42.5	14.5
	g平均	2198	54.7	15.2	g平均	2358	56.5	18.7	g平均	1922	46.6	16.8

	k コンドロイチン硫酸群 (g)				l Vitamine B_{12} 群 (g)			
	No	d体重	e肝重量	f心臓重量	No	d体重	e肝重量	f心臓重量
h二ヶ月	F 6	1500	36.2	9.0	L 6	1620	43.5	11.0
	5	1480	38.1	9.1	7	1800	42.5	9.8
	12	1500	34.0	10.2	8	1420	46.2	12.5
	13	1900	47.3	18.2				
	g平均	1595	38.9	11.6	g平均	1613.3	44.1	11.1
i三ヶ月	F 2	1750	37.1	14.0	L 2	1100	39.5	6.4
	7	1750	43.1	13.0	3	2000	42.0	13.5
	8	2010	40.5	17.0	12	2060	59.0	12.0
	10	1560	39.0	16.2	13	2180	48.5	14.0
	g平均	1767.5	39.9	15.1	g平均	1835	47.	11.5
j四ヶ月	F 1	1980	36.5	17.0	L 1	2500	64.0	18.0
	3	1730	33.5	15.0	4	2040	54.0	13.0
	4	2500	45.2	15.2	5	2100	37.0	17.0
	14	1420	40.5	9.8	15	1980	46.0	12.0
	g平均	1907.5	38.8	14.3	g平均	2155	50.0	15.0

Keys: a, methionine group; b, choline group; c, inositol group; d, body weight; e, liver weight; f, heart weight; g, average; h, 2nd month; i, 3rd month; j, 4th month; k, chondroitin sulfate group; l, vitamin B_{12} group;

TABLE 8. CHANGES IN BODY WEIGHT AND WEIGHT OF LIVER AND HEART (g)

a群	d月数	e体重	f肝重量	g心臓重量
b コレステロール群	2ヶ月h	1656	55.0	13.0
	3ヶ月i	2066	53.6	13.6
	4ヶ月j	2361	52.9	16.5
c対照群	2ヶ月h	1810	39.4	11.3
	3ヶ月i	2018	37.2	10.4
	4ヶ月j	2112	43.6	13.1

Keys: a, group; b, cholesterol group; c, control group; d, number of months; h, 2nd month; i, 3rd month; j, 4th month; e, body weight; f, liver weight; g, heart weight

TABLE 9. CHANGES IN THE SPECIFIC WEIGHT OF THE LIVER AND HEART

a群	i月数	j比肝重量 (肝重/体重)	k比心臓重量 (心臓重/体重)
b メチオニン	2ヶ月	0.025	0.0053
	3ヶ月	0.023	0.0059
	4ヶ月	0.025	0.0069
c コリン	2ヶ月	0.024	0.0076
	3ヶ月	0.023	0.0081
	4ヶ月	0.024	0.0079
d イノシット	2ヶ月	0.026	0.0067
	3ヶ月	0.021	0.0072
	4ヶ月	0.024	0.0087
e コ硫酸	2ヶ月	0.024	0.0073
	3ヶ月	0.023	0.0085
	4ヶ月	0.020	0.0075
f V.B_{12}	2ヶ月	0.027	0.0069
	3ヶ月	0.025	0.0065
	4ヶ月	0.023	0.0070
g 「コ」	2ヶ月	0.033	0.0079
	3ヶ月	0.026	0.0066
	4ヶ月	0.022	0.0070
h 対照	2ヶ月	0.022	0.0062
	3ヶ月	0.018	0.0052
	4ヶ月	0.020	0.0056

Keys: a, group; b, methionine; c, choline; d, inositol; e, chondroitin sulfate; f, vitamin B_{12}; g, cholesterol; h, control; i, month; j, relative weight of liver (liver/body weight); k, relative weight of heart (heart/body weight).

III. LIPID FRACTIONS IN THE LIVER

The measured values of the lipid fractions in the liver for individual groups are shown in Table 10. (The unit of measurement is mg/100 g, but will be abbreviated as mg in the following discussion.)

TABLE 10. LIVER LIPID FRACTIONS OF THE DRUG-TREATED GROUPS (mg/100 g)

a 群	i 脂質分画	j 二ヶ月	k 三ヶ月	l 四ヶ月
b メチオニン群	m 燐脂質	1905.6	1370.5	923.0
	n 総コレステリン	1725.8	1448.5	902.5
	o 遊離コレステリン	1381.0	934.5	234.5
	p エステルコレステリン	341.8	505.4	668.0
	q 中性脂肪	1425.0	1018.0	1433.0
	r 脂肪酸	3121.4	2542.0	3618.5
	s 総脂質	5300.2	4171.0	3672.5
	C / P	0.91	1.06	0.98
	E / T	0.18	0.35	0.74
		j 二ヶ月	k 三ヶ月	l 四ヶ月
c コリン群	m 燐脂質	2243.5	2016.5	1967.0
	n 総コレステリン	1754.5	1739.5	1795.0
	o 遊離コレステリン	872.0	775.5	1163.0
	p エステルコレステリン	882.5	964.0	632.0
	q 中性脂肪	1012.0	1210.5	1379.5
	r 脂肪酸	3107.5	2664.0	3119.5
	s 総脂質	5539.5	5564.0	5533.5
	C / P	0.78	0.86	0.91
	E / T	0.50	0.55	0.35
		j 二ヶ月	k 三ヶ月	l 四ヶ月
d イノシット群	m 燐脂質	2350.0	2080.0	1448.5
	n 総コレステリン	1055.5	1056.0	1259.5
	o 遊離コレステリン	816.0	775.5	979.0
	p エステルコレステリン	239.5	280.5	280.5
	q 中性脂肪	807.5	1444.0	1838.5
	r 脂肪酸	2797.0	3423.5	2680.5
	s 総脂質	4361.5	4754.0	4720.5
	C / P	0.45	0.51	0.87
	E / T	0.23	0.27	0.22
		二ヶ月	三ヶ月	四ヶ月
e コンドロイチン硫酸群	m 燐脂質	1704.0	2101.5	1604.5
	n 総コレステリン	1948.0	1809.5	1810.5
	o 遊離コレステリン	1178.5	1249.5	1214.0
	p エステルコレステリン	759.5	560.0	596.5
	q 中性脂肪	1618.0	1647.0	1947.5
	r 脂肪酸	2270.0	2775.0	3102.5
	s 総脂質	5731.0	5905.2	5732.5
	C / P	1.14	0.86	1.13
	E / T	0.93	0.31	0.33
		二ヶ月	三ヶ月	四ヶ月
f ビタミン B_{12} 群	m 燐脂質	1590.5	1668.5	1661.5
	n 総コレステリン	2150.5	2150.5	1820.0
	o 遊離コレステリン	1635.0	1529.0	1450.5
	p エステルコレステリン	515.5	621.5	369.5
	q 中性脂肪	1868.5	1364.5	2751.0
	r 脂肪酸	3191.5	4018.5	4895.0
	s 総脂質	5929.0	5569.0	6464.5
	C / P	1.35	1.29	1.10
	E / T	0.24	0.29	0.20
		二ヶ月	三ヶ月	四ヶ月
g コレステリン群	m 燐脂質	2212.4	2016.5	1930.5
	n 総コレステリン	2364.8	2320.5	2201.5
	o 遊離コレステリン	1750.6	1611.5	1171.5
	p エステルコレステリン	614.2	709.0	727.0
	q 中性脂肪	2227.0	2440.0	2849.8
	r 脂肪酸	1768.2	2020.0	2095.3
	s 総脂質	7185.8	7276.5	7332.6
	C / P	1.07	1.15	1.14
	E / T	0.26	0.31	0.33
		二ヶ月	三ヶ月	四ヶ月
h 対照群	m 燐脂質	2019.0	2023.5	1803.5
	n 総コレステリン	852.8	1081.0	902.5
	o 遊離コレステリン	410.2	540.5	250.5
	p エステルコレステリン	442.6	540.5	652.5
	q 中性脂肪	989.8	1130.0	1093.5
	r 脂肪酸	1645.0	2091.5	2640.5
	s 総脂質	4136.0	4569.5	4201.5
	C / P	0.42	0.53	0.49
	E / T	0.48	0.50	0.72

Keys: a, group; b, methionine group; c, choline group; d, inositol group; e, chondroitin sulfate group; f, vitamin B_{12} group; g, cholesterol group; h, control group; i, lipid fraction; j, 2nd month; k, 3rd month; l, 4th month; m, phospholipid; n, total cholesterine; o, free cholesterol; p, cholesterol ester; q, neutral fat; r, fatty acid; s, total lipid

(1) Phospholipid. The liver phospholipid level in the 2nd month when the body weight showed a decrease in response to the treatment at 1 g per kg of body weight of methionine, was 1905.5 mg, but the value dropped sharply to 1370.5 - 923.0 mg, approximately one half that of the control group, in the 3rd and 4th months when the dosage was 0.5 g. The phospholipid level of the choline group was similar to the cholesterol group in the 2nd, 3rd, and 4th months, slightly higher than the control level. As compared to the control, the phospholipid level of the inositol group was higher in the 2nd month, thesame in the 3rd month, and lower in the 4th month. The chondroitin sulfate group gave a value considerably lower than the control in the 2nd month, the same as the control in the 3rd month, and a lower value in the 4th month. Vitamin B_{12} group maintained a level considerably lower than the control, in the vicinity of 1600 mg

(2) Total cholesterol. The methionine group gave 1725.8 mg/dl, twice the value of the control, in the 2nd month, but the value dropped gradually in the 3rd and 4th months, to the control level in the 4th month. The value of the choline group in the 2nd month was twice that of the control, and subsequently settled in the vicinity of 1800 mg, beyond that of the control. The inositol group always maintained a value in the vicinity of 1100 mg, higher than the control yet approximately one half that of the cholesterol group. The total cholesterol of the chondroitin sulfate group in the 2nd to 4th months were twice that of the control group, but slightly lower than that of the cholesterol group. The total cholesterol level of the vitamin B_{12} group was in the 2100 mg range in the 2nd and 3rd months, but dropped slightly to 1820 mg in the 4th month, which is still twice that of the control.

Comparing the total cholesterol levels in the livers of the drug-treated groups with those of the control and cholesterol groups, all the drug-treated groupw maintained higher level. In the methionine group, a sharp increase occurred in the 2nd month, and a drop, to below the control level, in the 4th month. The choline group ranked between the control and the cholesterol group in total cholesterol level. The inositol group gave values close to those of the control throughout the experimental period. Increases shown by the chondroitin sulfate group were more notable than those exhibited by the choline group, and the vitamin B_{12} group reached a level close to that of the cholesterol group.

(3) Free cholesterol. The free cholesterol level of the methionine group became 4 times higher than the control level in the 2nd month, but a downward trend continued thereafter, finally returning to the level of the control in the 4th month. The choline group gave 872.0 and 775.0 mg in the 2nd and 3rd months, respectively, but exhibited a sharp rise, to 1163.0 mg, in the 4th month. The inositol group gave 816, 775, and 979 mg, in the 2nd, 3rd, and 4th months, respectively, which are considerably higher than the control values yet lower than that of the cholesterol group. The values shown by the chondroitin sulfate group were in the vicinity of 1200 mg. The vitamin B_{12} group also exhibited marked increases, but the values were lower than those of the cholesterol group.

Comparing with the control, all the drug-treated groups exhibited elevation in the liver free cholesterol level except for the valuesof the methionine group in the 4th month. The choline and inositol groups showed moderate elevation, and the chondroitin sulfate group a sharp increase, to 1200 mg, which, however, was still lower than that of the cholesterol group. The free cholesterol level of the vitamin B_{12} group also rose to a notably

high level, but did not quite surpass that of the cholesterol group.

(4) Cholesterol ester. The methionine group showed a value lower than the control in the 2nd month, but reached the latter's level in the 3rd month. The choline group gave a value of 882.5 mg, approximately twice that of the control, in the 2nd month, continued to show a rise in the 3rd month, and a drop to normal level in the 4th month. The inositol group maintained values in the vicinity of 200 mg throughout the experimental period, which is an obvious indication of suppressed cholesterol ester level. The chondroitin sulfate group gave a high 759.5 mg in the 2nd month, but the value returned to normal in the 3rd month. The cholesterol ester level of the vitamin B_{12} group also rose to 500 and 600 mg in the 2nd and 3rd months, respectively, but the value as of the 4th month was 369.5 mg.

In comparison with the control and cholesterol groups, the methionine group maintained a low level in the 2nd month, but showed increases in the 3rd and 4th months to levels comparable to that of the control, which, however, was still lower than that of the cholesterol group. The choline group surpassed the cholesterol group in liver cholesterol ester level, but a drop to the control level occurred in the 4th month. The inositol group maintained a level lower than the control at all times. The chondroitin sulfate group exhibited an increase in the 2nd month to a level slightly higher than that of the cholesterol group, but the values in the 3rd and 4th months were close to those of the control. The vitamin B_{12} group showed no significant difference from the control, but its value in the 4th month was slightly lower.

(5) Neutral fat. The methionine group exhibited fluctuations from 1425.0 mg in the 2nd month to 1048.0 mg in the 3rd month, and to 1433.0 mg in the 4th month. The neutral fat level of the choline group rose gradually from 1012.0 mg to 1379.5 mg in the 4th month. The value shown by the inositol group in the 2nd month was below the control value, but an increase occurred in the 3rd month, to 1838.5 mg in the 4th month. The chondroitin sulfate group exhibited an early elevation in the 2nd month, to 1618.0 mg, and a subsequent increase to 1947.0 mg in the 4th month. The vitamin B_{12} group gave 1868.5, 1364.5, and 2754.0 mg in the 2nd, 3rd, and 4th months, respectively, the last value being approximately equal to the value of the cholesterol group. In comparison with the control and the cholesterol group, the methionine and choline groups followed a pattern similar to that of the control. The value of the inositol group in the 2nd month was lower than the control value, but it showed a sharp increase in the 3rd month to a value twice that of the control. The value shown by the chondroitin sulfate group in the 2nd month was considerably higher than that of the control, andfurther elevation was noted in the 4th month. In general, the elevations shown by these drug-treated groups were still minimal as compared to that shown by the cholesterol group. The vitamin B_{12} group alone reached a level comparable to that of the cholesterol group.

(6) Fatty acid. The methionine group showed considerable fluctuations from a value of 3121.4 mg in the 2nd month to a lower level in the 3rd month, then to 3718.5 mg in the 4th month. Sharp increases were also noted in the choline group. The fatty acid level of the inositol group was also higher than the control, the values being within a range between 2680 and 3423 mg, and the chondroitin sulfate group continued to show an upward trend starting from 2270.0 mg to 3102.5 mg in the 4th month. The increases exhibited by the vitamin B_{12} group were most notable among those in the drug-treated groups, the values being almost twice that of the control.

As discussed in the previous report, the cholesterol group showed a level slightly higher than the control level in the 2nd month, but the value was lower thereafter. In comparison with the control and the cholesterol group, all the drug-treated groups exhibited extraordinary increases in fatty acid, the increase noted in the vitamin B_{12} group being most notable and that of the chondroitin sulfate group, least notable.

(7) Total lipid. The methionine group maintained a level higher than the control level in the 2nd month, with a value of 5300 mg, but a lower level thereafter. The choline group gave values in the vicinity of 5500 mg at all times, higher than the control value by approximately 1000 mg. The total lipid level of the insitol group fluctuated in the vicinity of 4000 mg, close to the control level. The chondroitin sulfate group indicated an intermediate degree of elevation, with 5700 - 5900 mg. The vitamin B_{12} group reached 6464.5 mg in the 4th month from 5929 mg in the 2nd month, the degree of elevation being highest among those of the drug-treated groups.

In comparison with the control and cholesterol group, the methionine group indicated a slight increase in the 2nd month, but the increase was temporary only to drop beyond the control level after the 3rd month. As compared to the cholesterol group, its lipid pattern indicated considerable suppression. The choline and chondroitin sulfate groups exhibited intermediate rates of elevation, and so did the vitamin B_{12} group although its actual level was slightly higher than that of the choline group. The inositol group showed fluctuations in the vicinity of 4000 mg throughout the experimental period, the pattern resembling that of the control group. In all cases, the total cholesterol level was below that of the cholesterol group, and the total lipid pattern indicated definite inhibition by these drugs within a range of variations.

(8) C/P ratio. The C/P ratio of the methionine group is approximately twice that of the control, but somewhat lower than that of the cholesterol group. The choline group gave a ratio slightly lower than that of the methionine group. The C/P ratio of the inositol group was lowest among the drug-treated groups, the values up to the 3rd month being approximately equal to the control value and slightly higher in the 4th month, although the values were still lower than those of other drug-treated groups. The chondroitin sulfate group gave values similar to those of the cholesterol group, considerably higher than those of the control. The C/P ratio of the vitamin B_{12} group was somewhat higher than that of the cholesterol group.

The C/P ratios in the liver shown by the inositol, choline and methionine groups were lower than that of the cholesterol group, and that of the chondroitin sulfate group, approximately equal to that of the latter. The vitamin B_{12} group showed a ratio higher than that of the cholesterol group.

(9) E/T ratio. As discussed in the previous report, the E/T ratio of the cholesterol group was lower than the control. The methionine group showed a ratio lower than that of the cholesterol group in the 2nd month, and higher in the 3rd and 4th months, the value in the 4th month being almost equal to the control value. The E/T ratio of the choline group was generally the same as the control value but slightly lower in the 4th month. The E/T ratio ofthe inositol group was always lowest among those of the drug-treated groups, approximately one half that of the control, and even lower than that of the cholesterol group. The chondroitin sulfate group exhibited a rate equal to that of the cholesterol group in the 3rd month.

The E/T ratio of the vitamin B_{12} group was lower than that of the cholesterol group and equal to that of the inositol group.

Thus, the E/T ratios of the choline and methionine groups were higher, that of the chondroitin sulfate group, almost equal to, and those of the inositol and vitamin B_{12} groups, lower than the value of the cholesterol group.

IV. DEGREE OF SCLEROTIC ALTERATION OF THE DRUG-TREATED GROUPS

The incidences of arteriosclerosis in aortic arch, thoracic aorta, and abdominal aorta following the drug treatments are tabulated in Table 11. The degrees of sclerotic alteration in average of the control group are shown by month in Table 12. In the methionine group, relatively strong

TABLE 11. INCIDENCES OF ARTERIOSCLEROSIS OF THE DRUG-TREATED GROUPS

	a メチオニン群					b コリン群					c イノシット群				
	No	d大動脈弓	e胸部大動脈	f腹部大動脈	g計	No	d大動脈弓	e胸部大動脈	f腹部大動脈	計g	No	大動脈弓d	e胸部大動脈	f腹部大動脈	g計
i 二ヶ月	C 2	2	1	1	4	D 1	2	1	1	4	E 2	0	0	1	1
	3	3	1	1	5	2	3.5	1	0	4.5	3	2	1	2	5
	6	0	1	0	1	3	1	1	1	3	10	1	1	1	3
	12	3	1	1	5	4	3	1	1	5	14	3	0	0	3
						6	2	0	2	4					
	h平均	2.0	1.0	0.75	3.75	平均h	2.3	0.8	1.0	4.1	h平均	1.5	0.5	1.0	3.0
j 三ヶ月	C 4	1	1	0	2	D 7	0	0	0	0	E 1	5	1	4	10
	10	3	1	2	6	9	3	2	4	9	5	1	1	1	3
	11	2	2	2	6	10	4.5	2	3	9.5	12	1	0	0	1
	14	1	0	1	2	11	2	1	3	6	13	1	0	0	1
	15	1	0	1	2	12	2	3	1	6	15	2	1	1	4
	平均h	1.6	0.8	1.2	3.6	h平均	2.3	1.6	2.2	6.1	平均h	2.0	0.6	1.2	3.8
k 四ヶ月	C 5	0	0.5	1	1.5	D 5	0	1	2	3	E 4	2	0	3	5
	7	4	1	4	9	8	1	1	1	3	7	3	1	2	6
	8	1	0	2	3	13	0	0	2	2	8	3	1	2	6
	9	1	0.5	1	2.5	14	1	1	1	3	9	0	1	2	3
	13	2	0	1	3	15	2	0	1	3	11	0	1	2	3
	平均h	1.6	0.4	1.8	3.8	平均h	0.8	0.6	1.4	2.8	平均h	1.6	0.8	2.2	4.6
	総平均l				3.7	総平均l				4.3	総平均l				3.9

	m コンドロイチン硫酸群					n Vitamine B_{12} 群				
	No	大動脈弓d	e胸部大動脈	f腹部大動脈	g計	No	d大動脈弓	e胸部大動脈	f腹部大動脈	g計
i 二ヶ月	F 5	2	1	1	4	L 6	3	1	2	6
	6	2	0.5	2	4.5	7	2	3	3	8
	12	3	1	2	6	8	3	3	2	8
	13	1	1	2	4					
	h平均	2.0	0.9	1.75	4.6	h平均	2.7	2.3	2.7	7.3
j 三ヶ月	F 2	3	1	3	7	L 3	3	2	2	7
	7	0	1	0	1	2	3	2	3	8
	8	3	0	3	6	12	3	2	3	8
	10	1	1	1	3	13	4	3	5	12
	h平均	1.8	0.8	1.8	4.25	h平均	3.25	2.25	3.25	8.75
k 四ヶ月	F 1	2	0	4	6	L 1	4	2	3	9
	3	3	1	2	6	4	2	2	2	6
	4	1	6	5	12	5	3	2	4	9
	14	3	1	2	6	15	3	1	2	6
	h平均	2.3	2.0	3.3	7.5	h平均	3.0	1.75	2.75	7.5
	総平均l				5.5	総平均l				7.9

Keys: a, methionine group; b, choline group; c, inositol group;

d, aortic arch; e, thoracic aorta; f, abdominal aorta; g, total; h, average; i, 2nd month; j, third month; k, 4th month; l, total average; m, chondroitin sulfate group; n, vitamin B_{12} group;

TABLE 12. THE DEGREES OF SCLEROTIC ALTERATION IN AVERAGE OF THE CONTROL GROUPS

a 月	b 大動脈弓	c 胸部大動脈	d 腹部大動脈	e 平均
f コレステロール群	2ヶ月k	3.2	2.0	3.4	8.6
	3ヶ月i	4.6	3.6	3.6	11.8
	4ヶ月j	4.5	2.8	3.0	10.2
	総平均k				10.2
g 対照群	2ヶ月h	0	0	0.7	0.7
	3ヶ月i	0	0.6	0.8	1.4
	4ヶ月j	0.2	0.2	0.2	1.2
	総平均l				1.1

Keys: a, month; b, aortic arch; b, thoracic aorta; d, abdominal aorta; e, avrage; f, cholesterol group; g, control group; h, 2nd month; i, 3rd month; j, 4th month; k, total average.

sclerotic alteration was noted in the arch of the aorta in the 2nd month, and sclerotic manifestations of relatively high severity began to appear from the 3rd month. However, on an overall basis, the extent of alteration was constant throughout a period from the 2nd to the 4th months. Th overall average during the experimental period was 3.7. The choline group indicated strong sclerotic alteration in aortic arch in the 2nd montn, but the changes in the abdominal and thoracic aortas increased severity in the 3rd month. It was also noted that the changes in aortic arch, abdominal aorta and thoracic aorta exhibited signs of alleviation in the 4th month. In general, the sclerotic alteration of low severity appearing in the 2nd month became aggravated in the 3rd month but alleviated in the 4th month. The overall average of the degree of sclerosis was 4.3. The inositol group developed sclerotic changes first in aortic arch, then in the abdominal aorta, but in the 3rd month, the alteration was of low severity with the exception of one case whose changes remained to be quite severe. In the 4th month, somewhat severe sclerotic changes were noted in abdominal aorta, but the overall average was 3.9, approximately equal to that of the methionine group. The chondroitin sulfate group indicated pronounced sclerotic alteration in aortic arch and abdominal aorta in the 2nd month, which became aggravated in the 4th month. The alteration in the abdominal aorta was particularly severe. The changes in this group became increasingly severe with time. The overall average was 5.5, second highest, next to that of the vitamin B_{12} group, among those of the drug-treated groups. For the vitamin B_{12} group, sclerotic changes of high severity began to appear in aortic arch, thoracic aorta and abdominal aorta early in the 2nd month, and became aggravated in the following months to a degree of 8.8. In this group, the sclerotic tendency was extremely strong throughout the experiment, with an overall average of 7.9, highest among those of the drug-treated groups. In comparison with the control and the cholesterol group with 1.1 and 10.2, respectively of sclerotic degree, the methionine and

inositol groups exhibited mild sclerotic alteration, the choline group, slightly more severe, and the chondroitin sulfate group, severer than the first three groups. The vitamin B_{12} group developed sclerosis of the highest severity among the drug-treated groups. In the methionine, inositol, choline, and chondroitin sulfate groups, the changes occurred first in aortic arch, spreading to abdominal and thoracic aorta. In the vitamin B_{12} group, pronounced sclerotic degeneration was already present in the 2nd month not only in aortic arch but in abdominal and thoracic aortas as well. Generally, the sclerotic alteration in the drug-treated groups was of high severity, higher than that of the control but lower than that of the cholesterol group.

V. CORRELATION BETWEEN THE DEGREE OF SCLEROSIS AND THE LIPID PATTERN IN THE LIVER AND BLOOD

The correlation between the degree of sclerosis and the lipid pattern in the liver and blood is shown in Table 13. The blood lipid level is expressed in terms of the average over a period of 2 to 16 weeks after the drug treatment, and the liver lipid level, the average from the autopsies in the 2nd, 3rd, and 4th months. The degree of sclerotic alteration is expressed in terms of an average of 3 months.

Phospholipid. The groups which showed no increase in blood phospholipid level indicate milder arteriosclerosis whereas the groups with marked elevation revealed sclerosis of high severity. No specific relation was noted between the phospholipid pattern in the liver and the degree of sclerotic alteration. The changes in blood phospholipid and liver phospholipid were not necessarily parallel.

Total cholesterol. The groups without sharp increase in total blood cholesterol indicated milder sclerotic alteration. The same tendency was observed in relation to the liver total cholesterol level. In the methionine and inositol groups, changes in total blood cholesterol did not exhibit a parallel relationship to total liver cholesterol. Generally speaking, however, the total cholesterol level appearred to be correlated to the degree of sclerotic alteration.

Free cholesterol. As total cholesterol, the level of free cholesterol in both blood and liver showed a correlation with the severity of sclerosis a higher level resulting in sclerosis of higher severity. The amount of free cholesterol in the blood was proportional to that in the liver.

Cholesterol ester. The amount of cholesterol ester in the blood or liver exhibited no specific tendency in relation to the severity of sclerotic alteration. Since the cholesterol ester levels of the methionine, choline, and chondroitin sulfate groups are lower than that of the cholesterol group, those with milder sclerotic change may seem to give lower rate of elevation in cholesterol ester level, but no generalization is permitted as is indicated by the fact that the inositol group, for instance, exhibited an increase at a comparable rate to that of the cholesterol group. No specific relationship was noted between the liver cholesterol ester level and the degree of sclerosis.

Neutral fat. No significant connection was observed between the blood neutral fat level and the severity of sclerosis, but the following correlation was present between the neutral fat level in the liver and the severity of sclerosis. When the increase in the liver neutral fat level was notable, the sclerotic alteration was of high severity, whereas with lower rate of elevation, the changes were milder. The changes in theneutral fat in the blood and the liver did not follow a parallel pattern.

Fatty acid. The cholesterol group exhibited an increase of fatty

TABLE 13. THE CORRELATION BETWEEN THE LIPID LEVELS IN THE BLOOD (mg/dl) AND THE LIVER (mg/100 g) AND THE SEVERITY OF SCLEROSIS.

a 群		b メチオニン	c コリン	d イノシット	e 硫酸	f V. B_{12}	g「コ」	h 対照
i 血液脂質 (mg/dl)	k 燐脂質	164.5	185.3	188.5	211.7	302.5	435.4	178.1
	l 総コレステリン	196.3	220.0	285.5	303.6	397.1	472.0	163.5
	m 遊離コレステリン	145.1	115.8	128.5	174.8	209.5	312.6	114.9
	n エステルコレステリン	51.3	104.2	156.9	129.0	187.1	161.3	49.4
	o 中性脂肪	519.8	576.8	410.1	378.2	414.7	475.0	322.8
	p 脂肪酸	565.1	522.3	577.8	541.7	641.8	726.2	359.5
	q 総脂質	898.6	1047.8	987.5	979.4	1235.0	1483.7	695.7
	C / P	1.20	1.22	1.61	1.44	1.42	1.13	0.93
	E / T	0.27	0.43	0.56	0.44	0.46	0.34	0.28
j 肝脂質 (mg/100gr)	k 燐脂質	1400.0	2076.0	1959.7	1803.7	1640.4	2053.0	1949.0
	l 総コレステリン	1359.3	1763.3	1123.7	1856.0	2040.7	2295.7	945.7
	m 遊離コレステリン	850.3	937.0	857.0	1214.0	1538.3	1611.3	400.7
	n エステルコレステリン	506.3	826.2	267.0	672.0	502.3	683.3	545.3
	o 中性脂肪	1302.0	1200.7	1363.7	1737.7	1995.7	2506.0	1071.3
	p 脂肪酸	3094.0	3064.0	2967.3	2716.0	4035.0	1961.0	2126.0
	q 総脂質	4414.7	5527.3	4612.3	5789.4	5987.7	7278.3	4303.3
	C / P	0.98	0.85	0.61	1.04	1.25	1.12	0.48
	E / T	0.43	0.47	0.24	0.34	0.24	0.30	0.57
r 硬化度		3.7	4.3	3.9	5.5	7.9	10.2	1.1

Keys: a, group; b, methionine; c, choline; d, inositol; e, chondroitin sulfate; f, vitamin B_{12}; g, cholesterol; h, control; i, blood lipid; j, liver lipid; k, phospholipid; l, total cholesterol; m, free cholesterol; n, cholesterol ester; o, neutral fat; p, fatty acid; q, total lipid; r, severity of sclerosis.

acid in the blood to a level approximately twice that of the control, but no increase in the liver fatty acid level was shown. The blood fatty acid levels of the drug-treated groups were between that of the cholesterol and the control level except for the vitamin B_{12} group which revealed severe sclerotic alteration and showed an increase to a level close to that of the cholesterol group. The rates of increase shown by the drug-treated groups were more significant than those of the control or the cholesterol group, that of the vitamin B_{12} group in particular being twice that of the control. The drug-treated groups generally showed changes in fatty acid level in the liver parallel to that in the blood, but the cholesterol group failed to follow this pattern.

Total lipid. A definite correlation was noted between the blood or liver total lipid level and the severity of sclerosis. The groups with sharp elevation of the total lipid level revealed high degree of sclerotic alteration. However, the chondroitin sulfate group developed arteriosclerosis of an intermediate degree despite the low rate of increase in total blood lipid.

C/P ratio. No parallel pattern was observed between the C/P ratio in the blood and that in the liver. The drug-treated groups exhibited higher C/P ratio than the cholesterol group and the control, which denies

any specific correlation between the C/P ratio and the degree of sclerotic alteration. Except for the vitamin B_{12} group, the liver C/P ratios of the drug-treated groups were lower than that of the cholesterol group, but this is not sufficient to support a possible relationship between the liver C/P ratio and the severity of sclerosis.

E/T ratio. There was no parallel relationship between the blood E/T and the liver E/T, nor between the blood E/T and the degree of change. However, E/T is likely to contribute to the elucidation of the actio mechanism of ea h drug on the cholesterol fraction.

The results summarized above demonstrate definite correlations between the degree of sclerotic alteration and the amount of blood phospholipid, the total cholesterol level, the cholesterol level in the blood and liver, the amount of neutral fat in the liver, and the total lipid levels in the blood and the liver.

CONCLUSION AND DISCUSSION

As stated in the previous report, the author found that high-cholesterol feed caused the hardening of all segments of aorta in chikens and sharp increases of the lipids in the blood and liver. At the present, no reliable therapy is available for human arteriosclerosis, and dietary therapy is practiced as the best resort. Unfortunately, dietary therapy is not fully effective. According to Pateck [20], low-cholesterol diet merely reduces the serum cholesterol temporarily and is unable to prevent it to eventually return to the original high level. Thus, the cure of endogenous hyperlipidemia is extremely difficult. The authr contemplated on a possibility that the administration of a lipotropic agent along with controlled diet prevents the occurrence of arteriosclerosis or improve sclerotic alteration which is already in progress, and studied the effects of various drugs on the lipid pattern in the blood and liver.

There have been a large number of papers on the effect of such drugs on arteriosclerosis. Steiner [21,27] administered choline chloride to cholesterol-fed rabbits and observed its antisclerotic action. He also reported that the accumulation of lipids on the aorta or coronary artery was frequently observed in rats lacking in choline. Morrison [22] reportedly prolonged the average survival period of pateitns with coronary arteriosclerosis by means of several types of lipotropic agent. Hueper [23] employed lecithin as a colloidal stabilizer and successfully reduced the cholesterol level. Greuel [24] also observed a reduction in cholesterol following the administration of methionine. Scherber [25] also experienced similar effects of lipotropic agents. In his experiment using hends, Hermann [28-30] observed depression of the cholesterol levels in the blood, aorta and liver following the administration of choline chloride and methionine. Oji [31-33] experimentally produced lack of methyl group in mice, and noted increases in fat deposit in the liver and the blood cholesterol level. He also observed marked decreases in cholesterol and γ- and β-globulins in the hypertensive patients treated with methionine. Similar experimental facts can be found in the reports published by Tasaka [33], Oshima [31], Yagi [35], Kurita [37], and Tahara [36].

The author conducted the present experiment after determining the dosage on the basis of these experimental results.

1. EFFECTS OF METHIONINE

Methionine, a sulfur-bearing essential amino acid, possesses a

methylthio group (CH_3-S group). The methyl group (CH_3-group) readily undergoes transmethylation in vivo, forming a choline, which is then synthetized into lecithin. Lecithin is capable of preventing fatty liver by rapidly changing in the liver and transporting fat from the liver. Higasa /95/ interpreted its action mechanism as that the compound stimulates the function of reticulo-endothelial cells converting neutral fat into lipoid, thereby activating the oxidation mechanism in the liver on a secondary basis. Mann /39/ produced experimental arteriosclerosis in monkeys by giving methionine-free diet, and subsequently observed the alleviation of the condition by methionine. In the present experiment, the author added methionine at 1 g per kg of body weight to cholesterol-containing feed, but due to marked reduction in body weight in 6 weeks, the dosage was reduced to 0.5 g from the 8th week, as a result of which the body weight increased sharply. Of the blood lipid fractions, phospholipid showed no increase, and the total cholesterol level rose during the period when a decrease in body weight was noted, but returned to normal after the dosage was reduced to 0.5 g. The free cholesterol level exhibited a sharp, temporary elevation at the time when the dosage was reduced, but eventually returned to normal. The cholesterol ester level exhibited a downward, instead of upward, tendency. The cholesterol fraction revealed clear suppression. The neutral fat level rose and the fatty acid level also showed a slight increase. The total lipid level was also higher, but fluctuations were minimal as compared to those shown by the cholesterol group, indicating a possitive inhibitory effect of the compound on the accumulation of lipids. The C/P was higher than that of the cholesterol group, and the E/T, lower, the value being similar to the control value. The weights of the liver and heart exhibited suppressed growth when the dosage was 1 g per kg of body weight, but upon reduction in dosage, marked increases took place, elevating the relative weights of the organs for body weight. Of the lipid fractions in the liver, phospholipid showed a sudden drop. The cholesterol ester level remained the same as that of the control. The neutral fat content increased to a slight degree, but its deposition in the liver was clearly controlled, for which methionine is generally regarded as lipotropic. The fatty acid level was elevated to beyond the level of the cholesterol or control group. This may be attributed to increased oxidation in the liver, and the components of the fatty acid fraction needs to be analyzed. The total lipid level followed an upward pattern as the total cholesterol level in the earlier period when a loss in body weight was noted, but after the dosage was reduced, it dropped to the control level, indicating the inhibition of the accumulation of lipids in the liver. The C/P ratio is high, and the E/T ratio was similar to that of the control. The suppression of sclerotic alteration was also observed histologically. In the present experiment, a dosage of 1 g per kg of body weight resulted in loss of body weight and subsequent increase of the lipids in the liver, but upon reduction of the dosage to 0.5 g, the body weight took a sudden rise and the liver lipid level improved. With regard to this phenomenon, Harper /49/ stated that increased methionine or cystine in diet resulted in shortage of threonine and induced fatty liver. Ichihara /41/ also observed liver disorder due to large dose administration of methionine. The dosage of 1 g per kg of body weight used in the present experiment was obviously an excessive dose, and the elevated liver lipid level and loss of body weight are attributed to hepatic disturbance. The reduction in dosage was responded with marked decrease in liver lipids and increase in body wieght, which substantiates the adequacy of the second dosage. The increases in the

total blood cholesterol and free cholesterol in the 10th week is regarded as transitory changes due to the elimination of the fat deposited in the liver.

As stated above, methionine is assumed to suppress the rise in the liver and blood lipid levels as proposed by Hermann and Morrison /22/, and control sclerotic alteration to a marked degree.

II. EFFECTS OF CHOLINE

It has been reported that, in animals, P_{32}- and C_{14}-labelled phosphorylcholine is incorporated into lecithin /44-46/. Choline incorporated into lecithin is said to possess an oxidizing or stabilizing action on lipids, but Artom /47/ held that it directly stimulated the oxidation of fatty acid, and Byer /49/ employed it for a therapeutic purpose because of its role as a fat stabilizer in lecithin.

The administration of choline chloride produced an extraordinary gain in body weight, and as Treadwell /50/ reported that choline stimulated the growth of rat, its stimulatory effect on growth was clearly evidenced in this experiment. Of the lipid fractions in the blood, phospholipid remained without increase, and free cholesterol and total cholesterol showed minimal increases. Cholesterole ester exhibited a slight increase. The neutral fat level rose, but subsequently dropped. The increase shown by the fatty acid fraction was to a minimal degree. The total lipid level was elevated to a marked degree in a pattern similar to that of the methionine group. The cholesterol ester alone exhibited an upward tendency, and both neutral fat and total lipid attained the levels shown by the methionine group, but did not reach that of the cholesterol group. The C/P is similar to that of the methionine group, but the E/T ratio was elevated. The weights of the liver and heart were greater than those of the cholesterol group, no significant difference was noted in the liver weight/body weight ratio, and the heart/body weight ratio was higher. The phospholipid level in the liver exhibited an elevation, and so did the total cholesterol, free cholesterol, and cholesterol ester fractions. The neutral fat and fatty acid levels rose to a slight and marked degrees, respectively, and an increase in total lipid was also noted. The fractions other than fatty acid and phospholipid exhibited marked suppression. The notable increase in phospholipid in the liver projects a possibility that choline does not possess the same action as methionine, and although choline may be synthetized from methionine, the amino acid in methionine or other factor in the decomposition process may also contribute to the production of phospholipid in the liver. The C/P ratio of the liver is lower than that of the methionine group, and the E/T ratio, considerably lower. The inhibitory effect of choline on the lipid in the liver was weaker than that of methionine, but, as compared to the cholesterol group, the deposition of lipids was well controlled. Stamler /54/ observed in his 15-week experiment with chikens treated with cholesterol, choline, and inositol that the total cholesterol and total lipid levels of the choline group were lower than those of the cholesterol group, but the phospholipid level in the liver was higher whereas the phospholipid levels in other organs dropped considerably. This observation, which is in agreement with the results of this experiment, is related to the fact that choline participates in the synthesis of phospholipid in the liver. Studying the inhibitory effect on lipid synthesis in relation to the degree of sclerotic alteration, the degree of alteration in the 2nd and 4th months was similar to that of the methionine group, but was higher in the 3rd month. This is in agreement with the fact that the blood lipid level rose notably in the 2nd and 3rd

months, but gradually dropped subsequently. These experimental results seem to indicate that this compound exerts its effect after a prolonged use. There are many reports concerning the use of choline. Steiner /21,27/, Morrison /22/, and Harmann /28,30/ observed inhibitory effects of choline on arteriosclerosis, and the author's experiment also demonstrated marked suppression of the condition and the lipid level by this agent. Thus, choline is assumed to be an effective antiarteriosclerotic agent.

III. EFFECTS OF INOSITOL

The lipotropic action of inositol was first brought out by Gavin /52/, but its mode of action is yet undetermined. MacFarland /53/ maintained that inositol acts specifically on fatty liver caused by biotin, on which methionine or choline exerts no effect. According to Arrigo /54/, the inactivation of succinic dehydrogenase in the liver due to carbon tetrachloride or high-fat diet could not be improved by choline or methionine, but was normalized by inositol. This experimental fact clearly demonstrates the difference in lipotropic action between choline or methionine and inositol. Best /55/ maintained that the effect of inositol was weaker than that of choline or methionine, and Takahashi /42/ denied the efficacy of inositol for the treatment of fatty liver. Oji /65/ reported that methionine and betaine improved the blood lipid pattern whereas inositol exerted an adverse effect. The lack of positive action of inositol was also mentioned by Stamler /63/. On the other hand, Hermann /61/ and Dotti /62/ observed that inositol suppressed the elevation of blood total cholesterol and phospholipid levels, eventually controlling arteriosclerosis. Felch /102/ also demonstrated its positive effect on cardiac infarction and diabetes. Thus, this compound is evaluated differently by different investigators, and its action mechanism is likely to differ from that of choline or methionine. In the present experiment, the treatment with inositol increased the body weight and permitted the normal growth of the cockerels. Of the lipid fractions in the blood, phospholipid revealed clear inhibition, but the total cholesterol level was elevated. No rise in free cholesterol level was observed, but cholesterol ester exhibited a considerable rate of increase. The neutral fact level rose to a slight degree, the fatty acid level was elevated, and the total lipid level reached beyond that of the methionine group. The C/P and E/T rations exhibited clear increases as compared to those of other drug-treated groups. No significant change was resualted in the liver/body weight and heart/body weight rations. Of liver lipid fractions, the toa total cholesterol and free cholesterol levels were depressed, and cholesterol ester indicated a sharp drop, contrary to that in the blood. A gradual rise was shown by the neutral fat fraction, and the fatty acid level rose, but the total lipid level became depressed to a significant degree. The C/P ratio was elevated, but the E/T ratio was extremely low. The cholesterol ester in the liver exhibited a notable decrease, and the phospholipid fraction, an increase. These results seem to suggest that inositol increases the production of phospholipid in the liver as evidenced in the relationship between choline and lecithin, but the phospholipid level in the liver dropped as the liver total cholesterol and neutral fat fractions increased. The severity of sclerotic alteration increased with month, but the inhibitory effect was as notable as that of methionine. The C/P ratio is said to increase with the severity of arteriosclerosis, and this was confirmed in this experiment in which the cholesterol group gave a value higher than that of the control. In the drug-treated groups, the inositol group in particular, the rate was higher than that of the cholesterol group despite

their lower severity of arteriosclerosis. Thus, the C/T ratio is not a valid standard for the judgment of the severity of arteriosclerosis nor the therapeutic effect of a drug on the disease. Oshima [67] also held a negative attitude toward the clinical significance of C/P ratio, and the author also failed to recognize any correlation even in the whole blood analysis. This also applies to the E/T ratio. The difference in E/T seems to be derived from the difference in the action mechanism of the drug itself, and this in turn demonstrates the fact that inositol works in a different manner from methionine or choline.

According to the experimental results, inositol suppressed arteriosclerosis to a notable degree, and inhibited the elevation of lipids in the blood and liver. Thus, this compound is effective as an anti-arteriosclerotic agent. It must be noted, however, that its effect should not be judged in terms of the blood cholesterol level or C/P ratio only.

IV. EFFECTS OF CHONDROITIN SULFATE

Meyer [64] defined chondroitin sulfate as a type of mucosaccharide comprising chondrosamine and glucuronic acid, and possessing hexosamine, hexouronic acid and sulfate as its constituents, as other agents such as heparin, mucoitin sulfate, and hyaluronic sulfate. Its molecular weight is considered to be between that of hyaluronic acid and that of heparin. In vivo, this compound acts on tissue injuries or functional disorder in general colloidal or elastin systems in its original high molecular form. stimulating the function of total connective tissues, and its basic substances decomposed in vivo exert antitoxic and antiallergic action and control sulfur metabolism [60,66]. Kurita [37] observed that condroitin sulfate suppressed the rise in serum cholesterol which was produced by the administration of cholesterol, depressed the cholesterol ester level, and inhibited sclerotic alteration to a notable degree, but he also demonstrated that these effects did not originate in the glucuronic acid or sulfuric acid group formed by the decomposition of chondroitin sulfate. The inhibitory action of this compound is yet to be clarified, but, in view of the lipotropic effect of heparin, a compound having a similar structure, it may possess a similar action. Hahn [68] successfully treated dietary hyperlipidemia by the intravenous injection of heparin, and moses [69] similarly inhibited cholesterol-induced hyperlipidemia and successfully reduced the β-lipoprotein and Sf_{10-20} and SF_{24-100}. Graham [70] and Oshima [34] also reported similar experimental results. Constanides [71], Horlick [72], and Horita [78] administered heparin to cholesterol-fed chickens, and inhibited atheromatous degeneration. Kawamura [76], Oshima [66], Kurita [87], Suzuki [79], Donomae [77], and Matsumoto [78] also observed positive effect of chondroitin sulfate in the treatment of lipidemia, and on the alleviation and prevention of arteriosclerosis. In the present investigation, the weight gain appeared normal up to the 3rd month, when it suddenly stopped without decrease. Of blood lipid fractions, phospholipid indicated a gradual increase, total and free cholesterol, increases in the 14th week, neutral fat, a slight increase, and fatty acid, a sharp increase after the 14th week. The increase noted in the total lipid level after the 10th week was extraordinary. The C/P and E/T ratios were elevated. The relative heart weight was high, but the relative liver weight was low. The liver phospholipid level dropped slightly, but the total cholesterol, free cholesterol, and cholesterol ester levels in the liver indicated sharp increases. However, the cholesterol ester level dropped in the later part of the experiment.

The neutral fat level increased, and the fatty acid level also rose slightly. The rise in total lipid after the 10th week was notable. The C/P ratio was lower than that of the cholesterol group and the E/T ratio, higher. The progress of sclerotic alteration was suppressed but there was one case which indicated severe sclerotic changes in the 4th month. The elevation of lipid levels in the blood and liver was more notable than that in the previous three groups, and the severity of sclerosis was also higher. However, as compared to the cholesterol group, there were strong indications that the elevation of lipid level was inhibited and the severity of morbid condition controlled. The increase in body weight came to a halt, which was also observed by Kurita in his experiment with rabbits /37/. Kurita attributed this phenomenon to the enhanced metabolism by chondroitin sulfate, but in this experiment, it was accompanied by an increase in blood lipid fraction. The relationship between these two changes cannot be readily explained, and it is difficult to explain the increase in blood lipid fraction by the reason of enhanced metabolism alone. The blood C/P ratio also showed a rise at that time, but the degree of sclerosis remained without change. Possible increase in blood lipid and decrease in body weight should be taken into consideration in the prolonged administration of this drug.

As stated above, chondroitin sulfate was found to reduce the occurrence of arteriosclerosis to some degree, and control the elevation of blood and liver lipid levels. These effects, along with its anti-aging action by the reactivation of the substances found among connective tissues, seem to endorse the efficacy of this compound as an antisclerotic drug.

V. EFFECT OF VITAMIN B_{12}

There are many reports on the effect of various vitamins on arteriosclerosis /80-95/. The lipotropic action of vitamin B_{12} was first discovered by Drill /96/, but its mode of action is yet to be clarified. Vitamin B_{12} bonds with α-globulin, and its effect is related to the synthesis of active methyl group /101/. The most unique characteristic of vitamin B_{12} is its inclusion of CO, which stimulates methionine metabolism, phosphoric acid metabolism, and production of A.T.P. while participating in the regeneration of liver parenchymal cells and formation of nucleic acid. Higasa /95/ stated that the combined use of methionine and vitamin B_{12} promoted the conversion of fat into lipoid to a more notable degree than the use of methionine alone. Endo /99/ and Chiyotani /100/ noted that vitamin B_{12} reduced the excretion of methionine and stimulated its transmethylation. According to Okuda /74/, the aged who possessed a large amount of vitamin B_{12} in his serum indicated a low serum cholesterol level. In his measurement of the liver lipid level in experimental cirrhosis, Takahashi /43/ observed stronger effect of this vitamin than methionine, and in Fujita's large dose administration experiment of vitamin B_{12} with subjects suffering from cerebroarteriosclerosis /48/, remarkable improvement was produced in the central artery of retina. In the present investigation, the administration of vitamin B_{12} permitted normal gain in body weight and favorable growth. Shimasono /26/ reported that vitamin B_{12} improved the growth of rat. In the author's experiment, the growth was gradual unlike that of the choline group.

Of the blood lipid fractions, the phospholipid, total and free cholesterol, and cholesterol ester levels all indicated marked increases, the neutral fat level, a slight elevation, and the fatty acid level, a notable rise followed by a slight decrease. The total lipid level was also elevated to a significant degree. The C/P and E/T ratios became higher

but the relative liver and heart weights merely showed slight increases. The phospholipid in the liver showed a slight drop. The increases in total and free cholesterol levels were notable, but the cholesterol ester fraction maintained normal level. There were marked increases in neutral fat and fatty acid. The total lipid of this group showed the highest rate of increase among the drug-treated groups but did not reach the level of the cholesterol group. The liver C/P ratio increased but the E/T ratio decreased.

As summarized above, the blood lipid and liver lipid levels indicated sharp elevation, and on an overall basis, the inhibitory capacity of vitamin B_{12} on hyperlipidemia and cholesterinosis is not as effective as expected. Its inhibition of arteriosclerosis is clearly weaker than that by other drugs, but, as compared to the cholesterol group, the vitamin B_{12} group exhibited signs of slight suppression. It was noted that, generally, the lipotropic action of vitamin B_{12} was weak and the control of sclerotic alteration by this agent was minimal. Thus, the administration of this compound alone was concluded as ineffective.

VI. RELATIONSHIP BETWEEN THE BLOOD AND LIVER LIPID FRACTIONS AND THE DEGREE OF SCLEROTIC ALTERATION

It is difficult to judge the effect of the therapy using these agents. In such an attempt, the serum cholesterol level, the serum C/P ratio or the lipoprotein factor is used as an index although the use of any single factor has proved inadequate for this purpose. These factors are not always valid in the study of the entire lipid metabolism, and the difference in the action mechanism of individual drugs and the dosage must also be taken into consideration.

Studying the blood and liver lipid fractions in relation to the severity of sclerosis, the blood phospholipid level indicated suppressed elevation in the group with milder sclerotic alteration. On the other hand, the liver phospholipid level followed no specific pattern. Both total and free cholesterol levels in the blood and liver are deeply related to the severity of sclerosis. When decreases were noted in the liver total cholesterol and phospholipid levels, the sclerotic alteration was less severe whereas, when a decrease was noted in liver phospholipid fraction but an increase occurred in liver total cholesterol, the sclerotic change was of higher severity. It appeared that the cholesterol ester level fluctuated according to the action mechanism of the drug. Such fluctuation exhibited no constant pattern, and that in the blood showed no correlation to that in the liver. The blood neutral fat level revealed no relationship with the degree of sclerotic alteration, but the group which indicated a suppressed neutral fat level of the liver showed milder changes. This suggests a possiblity that a drug with strong lipotropic action prevents neutral fat to be deposited in the liver and controls sclerotic alteration as well. The fatty acid level in the blood indicated controlled elevation when the degree of sclerosis was higher, and the liver fatty acid level showed an elevation in all drug-treated groups, which is indicative of the fact that the liver plays an important role in the oxidation process of fat, and that the lipotropic compound stimulated the oxidation. The total lipid level in the liver or blood is closely related to the severity of sclerosis. The correlation was particularly notable in the case of the liver.total lipid level. However, the C/P and E/T ratios are not necessarily correlative to the severity of sclerotic alteration.

Thus, the total and free cholesterol levels, and the total lipid levelsin the blood and liver are assumed to influence the degree of sclerotic alteration. The blood phospholipid, liver neutral fat and blood fatty acid levels also exhibit correlation to the severity of sclerosis, but the liver fatty acid level showed changes contradictory to the above observation. The action in which these drugs control arteriosclerosis is yet to be clarified, but, in view of the complexity of their action mechanism, no single fraction is sufficient to determine the effect of these drugs.

SUMMARY

Cholesterol-fed cockerels were divided into 5 groups, and methionine, choline, inositol, chondroitin sulfate and vitamin B_{12} were administered. The effects of these drugs on the incidence of atheromatous arteriosclerosis were examined, and changes in blood and liver lipid fractions studied. The results of the experiment are summarized below.

1. Methionine and inositol clearly suppressed athermatous arteriosclerosis. The suppressive action of chondroitin sulfate and choline was slightly weaker than that of the first two, and that of vitamin B_{12}, minimal.

2. Methionine notably suppressed the elevation of liver and blood lipid levels, excluding the neutral fat in the blood, and choline produced similar effect only to a lesser degree. The administration of inositol resulted in increased blood cholesterol ester, but inhibited the accumulation of other fractions in the blood and liver. Chondroitin sulfate also exerted inhibitory effects on the lipids in the blood and liver, but only to a slight degree. Vitamin B_{12} exhibited an inhibitory effect on the elevation of liver and blood lipid levels, but the effect was slight. Vitamin B_{12} exerted minimal suppressing action on the elevation of lipids in the blood and liver. The elevation of the liver fatty acid level following the administration of these drugs surpassed the level of the cholesterol group.

3. Of liver and blood lipid fractions, the total cholesterol, free cholesterol, and total lipid levels exhibited some sort of correlation with the degree of sclerosis. The group which indicated controlled sclerotic alteration also revealed inhibition of the increase of these fractions. The blood phospholipid level, the liver neutral fat level, and the blood fatty acid level exhibited similar tendencies.

4. Neither C/P nor E/T of the blood and liver indicated correlation with the severity of sclerosis. The blood C/P of the drug-treated groups were elevated, and the liver C/P of milder sclerotic cases were lower.

5. Methionine, inositol, choline, and chondroitin sulfate seem to be effective for the control of experimental atheromatous arteriosclerosis in chickens, but the single administration of vitamin B_{12} was found without effect.

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(Translator's note: The translation of Japanese titles is not provided due to illegibility.)